USADA and Floyd Landis
AAA No. 30 190 00847 06

United States Anti-Doping Agency's List of Exhibits*

Tab	Exhibit	Bates Numbers
1.	UCI Rules	
2.	Bylaws of the United States Olympic Committee	
3.	USADA Protocol for Olympic Movement Testing	
4.	World Anti-Doping Code	
5.	WADA 2006 Prohibited List	
6.	International Standard for Testing	
7.	Olympic Movement Anti-Doping Code	
8.	International Standard for Laboratories	
9.	TD2004EAAS	
10.	TD2004MRPL	
11.	TD2003LDOC	
12.	TD2003IDCR	
13.	USADA v. Hartman	
14.	Susin v. FINA	
15.	IAAF v. CBAt and Dos Santos	
16.	WADA v. Wium	
17.	IAAF v. Czech Athletic Federation and Roman Zubek	
18.	UCI v. Moller	

*USADA reserves the right to supplement these exhibits with any of the following materials: documents pertaining to the further analysis of Mr. Landis's other samples; documents related to discovery requests to Mr. Landis that are still pending; and any additional rebuttal exhibits necessary after review of Respondent's brief and exhibits, including any relevant demonstrative exhibits.

19.	UCI v. Bakker	
20.	UCI v. Skelde	
21.	UCI v. Danmarks	
22.	UCI v. Landaluce (English and French translations)	
23.	ISO/IEC 17025 (1999) and (2005)	USADA 1250-1318
24.	A Sample Laboratory Documentation Package	USADA 0001-0223
25.	B Sample Laboratory Documentation Package	USADA 0224-0370
26.	LNDD Documents	LNDD 0001-0519
27.	Respondent's Doping Control Form (CPLD Copy)	Produced by Landis
27a.	CPLD Copies of other riders doping control forms received by CPLD on July 27	
27b.	CPLD Copies of other riders doping control forms received by CPLD on July 28	
28.	Respondent's UCI License Application	USADA 1250-1252
29.	Longitudinal Study of Respondent's T/E Ratio	
30.	Longitudinal Study of Respondent's T/E Ratio Adjusted for Specific Gravity	
31.	Photograph of the type of cooler and metal band used to transport Respondent's sample	
32.	Color Photographs of LNDD operating the Isoprime instrument at 5E-6 mbar	
32a.	LNDD's Response to alleged IRMS Documentation Errors	
32b.	Documentation tracking sample 995474 CG-MS	
33.	Excerpt from Dr. Arnie Baker's PowerPoint Presentation	
34.	Cologne WADA Research Project Progress Report March 2007	
34a.	Cologne PowerPoint	

35.	UCLA Final Report on WADA Grant - Pharmacokinetics of Pharmaceutical Testosterone and TE in Subjects with Low and High Baseline TEs: The assessment of the ethnic differences and of the sensitivity of various carbon isotope ratio methods relative to TE, Don H. Catlin	
36.	UCLA Report to USADA on research subject IVT-1	
37.	Illustrations of Steroid Metabolism	
38.	Figures 1-21 from USADA Brief	
39.	Press Statements by Respondent • Sal Ruibal, *Synthetic testosterone found in Landis' 'B' sample; Tour winner likely to lose title,* USA TODAY, accessed Feb. 8, 2007, http://www.usatoday.com/sports/cycling/2006-08-04-landis-doping-test_x.htm • Associated Press, *Tour de France Champ Floyd Landis Requests Test of Backup Urine Sample in Doping Flap,* Fox News.com, Jul. 31, 2006, http://www.foxnews.com/printer_friendly_story/0,3566,206449,00.html • Sal Ruibal, *Landis fires back at cycling hierachy [sic],* USA TODAY, accessed Feb. 8, 2007, http://usatoday.printthis.clickability.com/pt/cpt?action=cpt&title=USATODAY.com+-+Lan...	

40.	Additional Science Articles	
	• *Validity of urine samples :microbial degradation. Recent advances in doping analysis*, Ayotte C., Charlebois, A., Lapointe, S., Barriault D. and Sylvestre M.	USADA 1154-1164
	• *Variability of T/E ratios in Athletes*, Baenziger J. and L. Bowers	USADA 1165-1170
	• *Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athlete's urine*, Catlin D. H., C. K. Hatton and S. H. Starcevic	USADA 1171-1179
	• *Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport,* Dehennin L. and A. M. Masumoto	USADA 1180-1190
	• *Evaluation of longitudinal studies, the determination of subject based reference ranges of the T/E ratio,* Donike M., S. Rauth, U. Mareck-Engelke, H. Geyer and R. Nitschke	USADA 1191-1194
	• *Stability of steroid profile (2) : excretion rates from morning urines,* Mareck-Engelke U., H. Geyer and M. Donike	USADA 1195-1197
	• *Improved method of detection of testosterone abuse by gas chromatography /combustion/ isotope ratio mass spectrometry analysis of urinary steroids,* Aguilera R., M. Becchi, H. Casabianca, C.K. Hatton, D. H. Catlin and B. Starcevic	USADA 1198-1205
	• *GC/C/IRMS and GC/MS in "Natural" Steroids Testing,* Ayotte C., D. Goudreault, A. Lajeunesse, M. Cléroux, Y. Richard, A. Charlebois, J. -P. Couture and A. Fakirian	USADA 1206-1211
	• *Kinetic isotope effects during metabolism of delta-4-steroids,* Flenker U. and Schanzer W	USADA 1212-1215
	• *Validity of carbon istope ration measurements for decomposed urine samples*, R. Taylor, A. Cawley, R. Kazlauskas, G. Trout & A. George	USADA 1216-1218
	• *Developments in Sports Drug Testing*; Trout, G., J. Rogerson; A. Cawley and C. Alma	USADA 1219-1224

	• *Performance Characteristics of a Carbon Isotope Ratio Method for Detecting Doping with Testosterone Based on urine Diols: Controls and Athletes with Elevated Testosterone/Epitestosterone Ratios*, Aguilera, R. T. Chapman, B. Starcevic, C. Hatton, D. Catlin	USADA 1225-1233
	• *Improved Detection of Sugar Addition to Maple Syrup Using Malic Acid as Internal Standard and in 13C Isotope Ratio Mass Spectrometry (IRMS)*, Tremblay, P. and R. Paquin	USADA 1234-1240
	• *Confirming Testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols*, Shackleton, C., A. Phillips, T. Chang and Y. Li	USADA 1241-1249

41.	USADA Documents	USADA 0371-0475
42.	USADA Documents	USADA 0476-0731
43.	USADA Documents	USADA 0732-0970
44.	USADA Documents	USADA 0971-1002
45.	USADA Documents	USADA 1003-1007
46.	USADA Documents	USADA 1008-1137
47.	USADA Documents	USADA 1138-1153
48.	Excerpt from Operating Manual for IsoPrime (Pg 103)	
49.	WADA Documents	WADA 0001-0143

TAB 27

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / Nationality of the person to be tested : USA

Conseil de prévention et de lutte contre le dopage — 27 JUIL. 2006

Adresse / Postal adress : 23356 BISHOP ROAD CA 92562 MURRIETA (USA)

N° de licence : 00202772

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14 10 1975

Sexe / Sex : M ☒ F ☐

Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒

Fédération – Discipline / Sport : UCI - FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 17ème ETAPE

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 20/07/2006

Heure de remise de la notification : 17 h 10

Lieu du contrôle / Place of test : MORZINE AVORIAZ UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : LANDIS
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : MERAVIGLIA GIOVANNI

Nom et prénom du médecin / Name of doctor : (en toutes lettres) BORDABERRY GERARD

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17 h 50

Date du prélèvement / Sampling date : 20/07/2006

Heure du prélèvement / Sampling time : 17 h 55

Identification / Identification :
Licence / Licence : ☒
Carte d'identité / ID : ☐
Passeport / Passport : ☐
Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 995474

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 125 ml

Densité / Specific Gravity : 1,030

pH / pH : 5

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n° | Deuxième n° / Second n° | Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)

05 05 06 Decanoate de testo 6 doses
05 05 06 Methylated inj 6 doses
22 07 06 Decanoate 2 inj
22 07 06 Methylated 1 inj
+ 05 de Prednisolone

AVT

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : Sac B scellé Urine → Protein +++

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Conseil de prévention et de lutte contre le dopage

IMPRIMERIE NATIONALE 6 002922 1

Produced by Landis

TAB 27 A

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested :

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :

Adresse / Postal adress :

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :
☐ Ministère chargé des Sports
☐ Direction régionale de la Jeunesse et des Sports
☐ Fédération nationale
☒ Fédération internationale

Conseil de prévention et de lutte contre le dopage
27 JUIL. 2006

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : Date de naissance / Birth Date : Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : VC1 - FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 17ème ETAPE

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 20/07/2006

Heure de remise de la notification : 17H10

Lieu du contrôle / Place of test : MORZINE AVORIAZ UNITÉ MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : / Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Delegate :

Nom et prénom du médecin / Name of doctor : (en toutes lettres) BORDABERRY GERARD

Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17H25

Date du prélèvement / Sampling date : 20/07/2006

Heure du prélèvement / Sampling time : 17H40

Identification / Identification :
Licence / Licence : ☒
Carte d'identité / ID : ☐
Passeport / Passport : ☐
Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994179

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 175 ml

Densité / Specific Gravity : 1,025

pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☐ OUI NON ☒

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Conseil de prévention et de lutte contre le dopage – 39, rue Saint-Dominique - 75700 Paris - Tél. : 01 40 62 76 76

CONFIDENTIAL

CONFIDENTIAL

6 002922 1

IMPRIMERIE NATIONALE

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :	Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :
Adresse / Postal adress : N° de licence :	Organisme demandant le contrôle / Organization requesting the test : ☐ Ministère chargé des Sports ☐ Direction régionale de la Jeunesse et des Sports ☐ Fédération nationale ☒ Fédération internationale Conseil de prévention et de lutte contre le dopage 27 JUIL. 2006
Nationalité / Nationality : Date de naissance / Birth Date : Sexe / Sex : M ☒ F ☐	Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒
Fédération – Discipline / Sport : UCI - FFC CYCLISME	Compétition : Internationale ☒ Nationale ☐ Régionale ☐ — Hors compétition : En club ☐ Stage hors club ☐ Cabinet médical ☐ Suivi ☐
Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 17ème ETAPE	

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date :	Heure de remise de la notification :	Lieu du contrôle / Place of test :
20/07/2006	17H10	MORZINE AVORIAZ VHITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature

Nom et prénom du médecin / Name of doctor (en toutes lettres) : BORDABERRY GERARD

Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival :	Date du prélèvement / Sampling date :	Heure du prélèvement / Sampling time :	Identification / Identification :
18H00	20/07/2006	18H05	Licence / Licence : ☒ Carte d'identité / ID : ☐ Passeport / Passport : ☐ Autre / Other : ☐

Échantillons A et B / Samples A and B — Code Flacon (et container) n° / bottle Code	Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml	Densité / Specific Gravity	pH / pH
994178	175 ml	1,030	5

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :	Refus / Refusal ☐ Oui ☐ Non	Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)	Commentaires sur la procédure / Comments on procedure :

6 002922 1

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.	Signature du Délégué Fédéral / Signature of Federal Delegate
	Signature du Médecin : / Signature of Doctor : Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

IMPRIMERIE NATIONALE

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested :

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :

Adresse / Postal adress :

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Conseil de prévention et de lutte contre le dopage
27 JUIL. 2006

N° de licence :

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : Date de naissance / Birth Date : Sexe / Sex : M ☒ F ☐

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Fédération – Discipline / Sport : UCI FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 17ÈME ET.

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date :	Heure de remise de la notification :	Lieu du contrôle / Place of test :
20-07-2006	16.30 h	MORZINE, WHITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signa

Nom et prénom du médecin / Name of doctor : (en toutes lettres) TOLLENAERE, BRUNO

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival :	Date du prélèvement / Sampling date :	Heure du prélèvement / Sampling time :
17-30 h	20-07-2006	18.15 h

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code

A	B
149976	270145

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 14 ML.

Densité / Specific Gravity : pH / pH :

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n° | Deuxième n° / Second n° | Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non — Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐ (AUT)

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delega

Signature du sportif contrôlé : Signature of person tested :	Signature du Médecin : Signature of Doctor :	Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

Exemplaire destiné au Conseil de prévention et de lutte contre le dopage – 39, rue Saint-Dominique - 75700 Paris - Tél. : 01 40 62 76 76

CONFIDENTIAL

CONFIDENTIAL

6 002922 1

IMPRIMERIE NATIONALE

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :

Adresse / Postal adress :

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Conseil de prévention et de lutte contre le dopage
27 JUIL. 2006

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : Date de naissance / Birth Date : Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : UCI FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 17 EME ET.

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 20-07-2006

Heure de remise de la notification : 16.30 h

Lieu du contrôle / Place of test : MORZINE, UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and sign... Delegate :

Nom et prénom du médecin / Name of doctor : (en toutes lettres) TOLLENAERE, BRUNO

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17.30 h

Date du prélèvement / Sampling date : 20-07-2006

Heure du prélèvement / Sampling time : 19.30

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994182

Quantité totale d'urine, ~~sang~~, ml / Total amount of urine, ~~blood~~, ml : 100 ml

Densité / Specific Gravity : 1.025

pH / pH : 6.0

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐ (AUT)

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

6 002922 1

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :	Signature du Médecin : / Signature of Doctor :	Signature de l'accompagnateur (eventuel) : / Signature of accompanying person (if any) :

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :

Adresse / Postal adress :

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :

☐ Ministère chargé des Sports
☐ Direction régionale de la Jeunesse et des Sports
☐ Fédération nationale
☒ Fédération internationale

Conseil de prévention et de lutte contre le dopage
27 JUIL. 2006

Type de contrôle / Type of test :

Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : GERMANY

Date de naissance / Birth Date : 09 09 1981

Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : VC1 – FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 17ème ETAPE

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 20/07/2006

Heure de remise de la notification : 17h10

Lieu du contrôle / Place of test : MORZINE AVORIAZ UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate :

Nom et prénom du médecin / Name of doctor : (en toutes lettres) BORDABERRY GERARD

Signature du médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17h25

Date du prélèvement / Sampling date : 20/07/2006

Heure du prélèvement / Sampling time : 17h30

Identification / Identification :
Licence / Licence : ☒
Carte d'identité / ID : ☐
Passeport / Passport : ☐
Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code

149986 270141

~~Quantité totale d'urine~~, sang, ml
~~Total amount of urine~~, blood, ml
14 ml

Densité / Specific Gravity

pH / pH

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☐ OUI NON ☒

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral
Signature of Federal Delegate

Signature du sportif contrôlé :
Signature of person tested :

Signature du Médecin :
Signature of Doctor :

Signature de l'accompagnateur (éventuel) :
Signature of accompanying person (if any) :

CONFIDENTIAL

6 002922 1

IMPRIMÉ EN FRANCE

TAB 27 B

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :

Adresse / Postal adress :

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Conseil de prévention et de lutte contre le dopage
28 JUIL. 2006

Type de contrôle / Type of test :

Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : Date de naissance / Birth Date : Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : UCI FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 16ÈME ET.

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 19-07-2006

Heure de remise de la notification : 16.45 h.

Lieu du contrôle / Place of test : LA TOUSSUIRE UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du ~~Délégué~~ Fédéral / Name and signature

Nom et prénom du médecin / Name of doctor : (en toutes lettres) TOLLENAERE, BRUNO

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17.50 h.

Date du prélèvement / Sampling date : 19-07-2006

Heure du prélèvement / Sampling time : 18.10 h

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994077

Quantité totale d'urine, ~~sang~~, ml / Total amount of urine, ~~blood~~, ml : 125 ml

Densité / Specific Gravity : 1.025

pH / pH : 6.0

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :	Refus / Refusal	Oui	Non	Empêchement / Obstacle	Oui	Non

Médicaments / Drugs ☒ OUI NON ☐ (AUT)

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

6 003922 1

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delega

Signature du sportif contrôlé : Signature of ~~person~~ tested :	Signature du Médecin : Signature of Doctor :	Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

IMPRIMERIE NATIONALE

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :	Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :
Adresse / Postal adress : N° de licence :	Organisme demandant le contrôle / Organization requesting the test : ☐ Ministère chargé des Sports ☐ Direction régionale de la Jeunesse et des Sports ☐ Fédération nationale ☒ Fédération internationale Conseil de prévention et de lutte contre le dopage 28 JUIL. 2006
Nationalité / Nationality : Date de naissance / Birth Date : / / Sexe / Sex : M ☒ ☐ Fédération – Discipline / Sport : UCI FF Cyclisme Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 16 EME ET	Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒ Compétition : Internationale ☒ Nationale ☐ Régionale ☐ Hors compétition : En club ☐ Stage hors club ☐ Cabinet médical ☐ Suivi ☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date :	Heure de remise de la notification :	Lieu du contrôle / Place of test :
19-07-2006	16.45 h	LA TOUSSUIRE UNITÉ MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of

Nom et prénom du médecin / Name of doctor : (en toutes lettres)	Signature du Médecin : Signature of Doctor :
TOLLENAERE, BRUNO	

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival :	Date du prélèvement / Sampling date :	Heure du prélèvement / Sampling time :	Identification / Identification : Licence / Licence : ☒ Carte d'identité / ID : ☐ Passeport / Passport : ☐ Autre / Other : ☒
17.50 h	19-07-2006	18.05 h	

Échantillons A et B / Samples A and B Code Flacon (et container) n° / bottle Code	Quantité totale ~~d'urine~~, sang, ml Total amount ~~of urine~~, blood, ml	Densité / Specific Gravity	pH / pH
149947 270146	14 ML		

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :	Refus / Refusal ☐ Oui ☐ Non	Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☐ OUI NON ☐ (AUT)

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)	Commentaires sur la procédure / Comments on procedure :

Commentaires sur la procédure / Comments on procedure

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.	Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :	Signature du Médecin : / Signature of Doctor :	Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

6 002922 1

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :

Adresse / Postal adress :

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Conseil de prévention et de lutte contre le dopage
28 JUIL. 2006

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : Date de naissance / Birth Date : Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : VCI - FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 16ème ETAPE

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 19/07/2006 — Heure de remise de la notification : 17 H 20 — Lieu du contrôle / Place of test : LA TOUSSUIRE UNITÉ MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate :

Nom et prénom du médecin / Name of doctor : (en toutes lettres) BORDABERRY GERARD

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17 H 30 — Date du prélèvement / Sampling date : 19/07/2006 — Heure du prélèvement / Sampling time : 17 H 45

Identification / Identification :
Licence / Licence : ☒
Carte d'identité / ID : ☐
Passeport / Passport : ☐
Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 149948 270139

~~Quantité totale d'urine~~, sang, ml
~~Total amount of urine~~, blood, ml : 14 ml

Densité / Specific Gravity :

pH / pH :

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

6 0023221

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral
Signature of Federal Delegate

Signature du sportif contrôlé :
Signature of person tested :

Signature du Médecin :
Signature of Doctor :

Signature de l'accompagnateur (éventuel) :
Signature of accompanying person (if any) :

IMPRIMERIE NATIONALE

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :

Adresse / Postal adress :

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :

☐ Ministère chargé des Sports
☐ Direction régionale de la Jeunesse et des Sports
☐ Fédération nationale
☒ Fédération internationale

Conseil de prévention et de lutte contre le dopage
28 JUIL. 2006

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : Date de naissance / Birth Date : Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : VCI - FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 16ème ETAPE

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 19/07/2006 — Heure de remise de la notification : 17H15 — Lieu du contrôle / Place of test : LA TOUSSUIRE UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate :

Nom et prénom du médecin / Name of doctor (en toutes lettres) : BORDA BERRY GERARD

Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 18 H 20

Date du prélèvement / Sampling date : 19/07/2006

Heure du prélèvement / Sampling time : 18 H 40

Identification / Identification :
Licence / Licence : ☒
Carte d'identité / ID : ☐
Passeport / Passport : ☐
Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 149938 270143

~~Quantité totale d'urine~~, sang, ml / ~~Total amount of urine~~, blood, ml : 14 ml

Densité / Specific Gravity :

pH / pH :

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :	Refus / Refusal	Oui ☐	Non ☐	Empêchement / Obstacle	Oui ☐	Non ☐

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

6 002922 1

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

IMPRIMERIE NATIONALE

Exemplaire destiné au Conseil de prévention et de lutte contre le dopage – 39, rue Saint-Dominique - 75700 Paris - Tél. : 01 40 62 76 76

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :	Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :
Adresse / Postal adress : N° de licence :	Organisme demandant le contrôle / Organization requesting the test : ☐ Ministère chargé des Sports ☐ Direction régionale de la Jeunesse et des Sports ☐ Fédération nationale ☒ Fédération internationale Conseil de prévention et de lutte contre le dopage 28 JUIL. 2006 Type de contrôle / Type of test :
Nationalité / Nationality : Date de naissance / Birth Date : Sexe / Sex : M ☒ F ☐ Inopiné : Oui ☐ Non ☒	
Fédération – Discipline / Sport : UCI - FFC CYCLISME	Compétition : Internationale ☒ Nationale ☐ Régionale ☐ — Hors compétition : En club ☐ Stage hors club ☐ Cabinet médical ☐ Suivi ☐
Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 16ème ETAPE	

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date :	Heure de remise de la notification :	Lieu du contrôle / Place of test :
19/07/2006	17H15	LA TOUSSUIRE UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate :

Nom et prénom du médecin / Name of doc... (en toutes lettres) BORDABERRY GERARD

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival :	Date du prélèvement / Sampling date :	Heure du prélèvement / Sampling time :	Identification / Identification : Licence / Licence : ☒ Carte d'identité / ID : ☐ Passeport / Passport : ☐ Autre / Other : ☐
18 H 20	19/07/2006	18 H 45	

Échantillons A et B / Samples A and B — Code Flacon (et container) n° / bottle Code	Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml	Densité / Specific Gravity	pH / pH
994183	150 ml	1,030	5

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :	Refus / Refusal ☐ Oui ☐ Non	Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

6 002922 1

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral
Signature of Federal Delegate

Signature du sportif contrôlé : Signature of person tested :	Signature du Médecin : Signature of Doctor :	Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

TARKETT NATIONAL

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :

Adresse / Postal adress :

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Conseil de prévention et de lutte contre le dopage
28 JUIL. 2006

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : Date de naissance / Birth Date : Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : UCI - FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 16ème ETAPE

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date :	Heure de remise de la notification :	Lieu du contrôle / Place of test :
19/07/2006	17h20	LA TOUSSUIRE UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate :

Nom et prénom du médecin / Name of docto (en toutes lettres) : BORDABERRY GERARD

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival :	Date du prélèvement / Sampling date :	Heure du prélèvement / Sampling time :
17h30	19/07/2006	17h50

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994181

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 125 ml

Identification / Identification :
Licence / Licence : ☒
Carte d'identité / ID : ☐
Passeport / Passport : ☐
Autre / Other : ☐

Densité / Specific Gravity : 1.030

pH / pH : 5

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :	Refus / Refusal	Oui	Non	Empêchement / Obstacle	Oui	Non
		☐	☐		☐	☐

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

6 002922 1

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :	Signature du Médecin : / Signature of Doctor :	Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

IMPRIMERIE NATIONALE

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

CONFIDENTIAL

Nom et prénom du Sportif / Name of person to be tested :

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested :

Adresse / Postal adress :

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Conseil de prévention et de lutte contre le dopage
28 JUIL. 2006

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality :

Date de naissance : / Birth Date :

Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : UCI FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 16 EME ET

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 19-07-2006

Heure de remise de la notification : 16.45 h

Lieu du contrôle / Place of test : LA TOUSSUIRE, UNITÉ MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signatu

Nom et prénom du médecin / Name of doctor : (en toutes lettres) TOLLENAERE, BRUNO

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17.40 h

Date du prélèvement / Sampling date : 19-07-2006

Heure du prélèvement / Sampling time : 17.50 h

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 995383

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 150 cc

Densité / Specific Gravity : 1,015

pH / pH : 6,0

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n° | Deuxième n° / Second n° | Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal : Oui ☐ Non ☐

Empêchement / Obstacle : Oui ☐ Non ☐

Médicaments / Drugs ☒ OUI NON ☐ (AUT)

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

6 002922 1

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

IMPRIMERIE NATIONALE

TAB 28

USA CYCLING

State of *Colorado*
County of *El Paso*

I certify that this is a true and correct copy of Floyd Landis's Applicant Declaration on file with USA Cycling.

Dated: April 12, 2007

Deborah A Francis
Notary

My commission expires: 1/16/11.

1 Olympic Plaza, Colorado Springs, CO 80909-5775, Phone: 719.866.4581, Fax: 719.866.4628, www.usacycling.org

APPLICANT DECLARATION

Name: Floyd A. Landis
Annual License#: 20272
RACING AGE (as of December 31, 2006): 31
Address: 23356 BISHOP RD
City: MURRIETA **ST:** CA **ZIP:** 92562
Phone: (909) 461-8462

Additional representations for International and USPRO Licenses
I am not aware of any reason why the requested license should not be issued. I have not requested a license from the UCI or from any other National Federation for the calendar year. I am solely responsible for the information contained in this application to the UCI, its Continental Federations, and its National Federations. I agree that the sole jurisdiction for resolving disputes that may arise shall be in the courts of the domicile of the UCI. By participating in a race where a drug test is conducted, in accordance with UCI, World Anti-doping Association (WADA) or U.S. Anti-Doping Agency (USADA) drug test regulations, I agree to submit to such testing. I further agree that when competing outside the United States or in international races the results of the analysis may be released to the public and communicated to my trade team, coach, or doctor in accordance with UCI and WADA regulations. I agree to allow my doctor and/or the doctor of my team, upon the request of the UCI or WADA, to release to UCI and WADA officials a list of medications or treatments administered to me before any specific competition. I agree to submit any protests or disputes regarding drug testing when competing outside the United States or in international races to the Court of Arbitration for Sports (CAS), whose decision I shall accept as final. I agree that all urine samples in such cases taken shall become the property of the UCI and WADA, and that UCI and WADA may have them analyzed for any purpose, including, without limitation, general research and information on health protection. I accept these conditions regarding testing and agree to undergo all tests required of me.

Signature of Applicant _____
Date 1-16-06

CONSENT AND AGREEMENT OF PARENT OR GUARDIAN

I am the parent or guardian of Floyd A. Landis (Child). I give permission for my Child to enter any event permitted or sanctioned by USA Cycling, another event national federations, or International Cycling Union (UCI) during the period of the license applied for. **I HAVE READ AND I UNDERSTAND THE ABOVE CONTRACT.** In consideration of allowing my Child to participate, I consent to the contract and agree that **ITS TERMS SHALL LIKEWISE BIND ME, MY CHILD,** my heirs, legal representatives, and assignees. **I HEREBY RELEASE AND SHALL DEFEND, INDEMNIFY AND HOLD HARMLESS THE RELEASEES FROM EVERY CLAIM AND ANY LIABILITY** that I or my Child may allege against the Releasees (including reasonable legal fees and costs) as a direct or indirect result of injury or death to me or my Child because of my Child's participation in a USA Cycling event, **WHETHER CAUSED BY THE NEGLIGENCE OF THE RELEASEES** or others. **I PROMISE NOT TO SUE RELEASEES** on my behalf or on behalf of my Child regarding any claim arising from my Child's participation in a USA Cycling event.

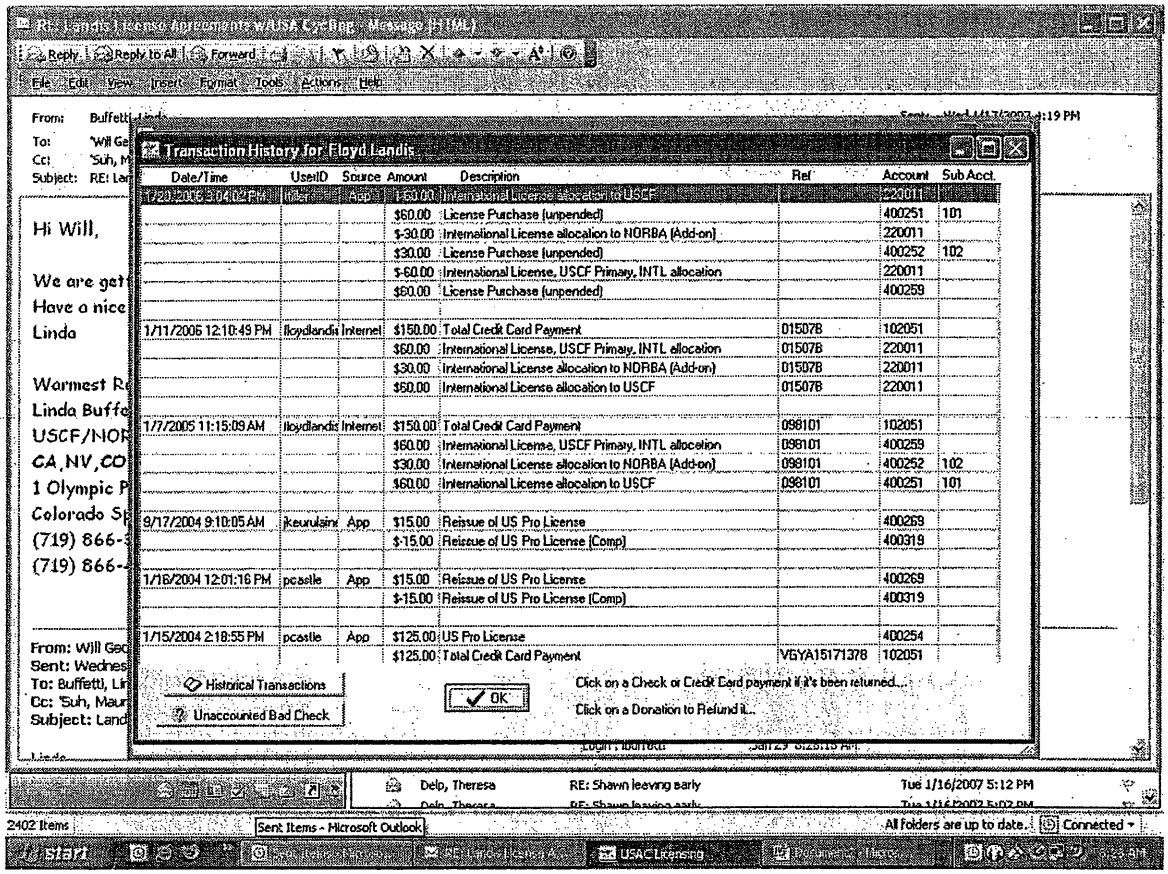
Signature of Parent or Guardian _____
Date _____

The above signed agreement should be returned to:

USA Cycling, Inc.
ATTN Membership Department
1 Olympic Plaza
Colorado Springs, CO 80909.

JAN 20 2006

Please note a minor's on-line application cannot be fully processed and the license cannot be issued until this signed agreement is received.


Transaction History for Floyd Landis
Date/Time UserID Source Amount Description Ref Account Sub Acct
1/20/2006 3:04:02 PM floyd App $60.00 International License allocation to USCF 220011
$60.00 License Purchase (unpended) 400251 101
$-30.00 International License allocation to NORBA (Add-on) 220011
$30.00 License Purchase (unpended) 400252 102
$-60.00 International License, USCF Primary, INTL allocation 220011
$60.00 License Purchase (unpended) 400259
1/11/2006 12:10:49 PM floydlandis Internet $150.00 Total Credit Card Payment 015078 102051
$60.00 International License, USCF Primary, INTL allocation 015078 220011
$30.00 International License allocation to NORBA (Add-on) 015078 220011
$60.00 International License allocation to USCF 015078 220011
1/7/2005 11:15:09 AM floydlandis Internet $150.00 Total Credit Card Payment 098101 102051
$60.00 International License, USCF Primary, INTL allocation 098101 400259
$30.00 International License allocation to NORBA (Add-on) 098101 400252 102
$60.00 International License allocation to USCF 098101 400251 101
9/17/2004 9:10:05 AM jkeurulain App $15.00 Reissue of US Pro License 400269
$-15.00 Reissue of US Pro License (Comp) 400319
1/16/2004 12:01:16 PM pcastle App $15.00 Reissue of US Pro License 400269
$-15.00 Reissue of US Pro License (Comp) 400319
1/15/2004 2:18:55 PM pcastle App $125.00 US Pro License 400254
$125.00 Total Credit Card Payment VGYA15171378 102051
Historical Transactions
Unaccounted Bad Check
OK
Click on a Check or Credit Card payment if it's been returned...
Click on a Donation to Refund it...
Delp, Theresa RE: Shawn leaving early Tue 1/16/2007 5:12 PM
2402 Items
Sent Items - Microsoft Outlook
All folders are up to date.
Connected
start
USAC Licensing

TAB 29

URINE RESULTS FROM WADA ACCREDITED LABS

Floyd Landis

Lab	Bottle#	Type	Collection	SG	TE	[Test]	[Test]cr	[Epi]	[Epi]cr	[Andr]	[Andro]cr	[Etio]	[Etio]cr	[5aAD]	[5aAD]cr	[5bAD]	[5bAD]cr	Andro/Etio	Andro/T
Paris	176593		06/16/02	1.023	1.30	28.0	24.3	21.0	18.3	1502	1306	1515	1317	27.0	23.5	184.0	160.0	1.0	53.6
UCLA	466193	OOC	12/08/02	1.020	1.13	16.1	16.1	15.0	15.0	1692	1692	1676	1676	45.0	45.0	169.0	169.0	1.0	105.1
Paris	808345		07/26/03	1.018	1.30	22.0	24.4	18.0	20.0	848	942	1267	1408	18.0	20.0	109.0	121.1	0.7	38.5
Madrid	336873		08/15/03	1.007	1.50	84.1	240.3	57.3	163.7	4111	11746	2951	8431	73.2	209.1	392.0	1120.0	1.4	48.9
UCLA	476315	OOC	11/26/03	1.023	1.10	14.0	12.2	14.7	12.8	859	747	1633	1420	37.0	32.2	153.0	133.0	0.5	61.4
Lisbon	277821	IC	02/22/04	1.011	1.00	19.6	35.6	18.9	34.4	887	1613	754	1371	18.9	34.4	39.1	71.1	1.2	45.3
Paris	289130	IC	03/09/04	1.018	1.30	22.0	24.4	19.0	21.1	1429	1588	1436	1596	25.0	27.8	88.0	97.8	1.0	65.0
Madrid	342599	IC	09/04/04	1.019	1.70	25.8	27.2	14.5	15.3	1761	1854	1902	2002	27.9	29.4	143.9	151.5	0.9	68.3
Madrid	342856	IC	09/11/04	1.014	2.00	7.5	10.7	3.7	5.3	395	564	438	626	5.8	8.3	18.4	26.3	0.9	52.7
Madrid	342850	IC	09/12/04	1.025	1.40	39.9	31.9	29.4	23.5	2605	2084	2702	2162	32.4	25.9	144.9	115.9	1.0	65.3
Madrid	348369	IC	09/13/04	1.019	1.30	25.5	26.8	19.4	20.4	1646	1733	1301	1369	22.3	23.5	153.1	161.2	1.3	64.5
Madrid	347883	IC	09/14/04	1.019	1.00	36.2	38.1	37.9	39.9	2529	2662	1991	2096	32.7	34.4	288.7	303.9	1.3	69.9
UCLA	485155	OOC	01/08/05	1.012	0.80	17.2	28.7	23.8	39.7	933	1555	1310	2183	30.0	50.0	98.0	163.3	0.7	54.2
UCLA	920460	IC	04/21/05	1.015	1.50	11.0	14.7	7.0	9.3	710	947	697	929	9.0	12.0	32.0	42.7	1.0	64.5
UCLA	920462	IC	04/22/05	1.025	1.20	24.0	19.2	21.0	16.8	1416	1133	1491	1193	30.0	24.0	110.0	88.0	0.9	59.0
Paris	874535*	IC	07/17/05	1.036	2.20	32.0	17.8	15.0	8.3	570	317	576	320	22.0	12.2	41.0	22.8	1.0	17.8
UCLA	491607	OOC	10/07/05	1.026	1.09	40.6	31.2	41.9	32.2	1898	1460	2459	1892	138.0	106.2	435.0	334.6	0.8	46.7
UCLA	493084	OOC	11/05/05	1.018	1.08	29.8	33.1	30.0	33.3	1367	1519	1668	1853	75.0	83.3	255.0	283.3	0.8	45.9
UCLA	493091	OOC	01/06/06	1.025	0.89	29.5	23.6	33.7	27.0	1284	1027	3985	3188	40.0	32.0	253.0	202.4	0.3	43.5
UCLA	951826	IC	02/22/06	1.026	1.80	28.0	21.5	16.0	12.3	1006	774	1148	883	18.0	13.8	86.0	66.2	0.9	35.9
UCLA	951875	IC	02/23/06	1.022	1.50	29.0	26.4	19.0	17.3	1222	1111	1283	1166	20.0	18.2	71.0	64.5	1.0	42.1
UCLA	951828	IC	02/24/06	1.025	1.50	29.0	23.2	20.0	16.0	796	637	1977	1582	26.0	20.8	137.0	109.6	0.4	27.4
UCLA	951866	IC	02/25/06	1.025	1.70	35.0	28.0	22.0	17.6	1621	1297	2079	1663	38.0	30.4	205.0	164.0	0.8	46.3
UCLA	951872	IC	02/26/06	1.016	1.80	20.0	25.0	12.0	15.0	1106	1383	1359	1699	14.0	17.5	85.0	106.3	0.8	55.3
Paris	876013	IC	03/08/06	1.027	1.50	65.0	48.1	46.0	34.1	2302	1705	2755	2041	39.0	28.9	202.0	149.6	0.8	35.4
Paris	874285	IC	03/09/06	1.022	1.50	37.0	33.6	27.0	24.5	1305	1186	1362	1238	26.0	23.6	129.0	117.3	1.0	35.3
Paris	872364	IC	03/10/06	1.022	1.30	39.0	35.5	31.0	28.2	1302	1184	1696	1542	28.0	25.5	167.0	151.8	0.8	33.4
Paris	872357	IC	03/11/06	1.024	1.60	61.0	50.8	41.0	34.2	1905	1588	2509	2091	55.0	45.8	308.0	256.7	0.8	31.2
Paris	876004	IC	03/12/06	1.016	1.60	36.0	45.0	24.0	30.0	1298	1623	1385	1731	28.0	35.0	152.0	190.0	0.9	36.1
UCLA	1504999	OOC	04/10/06	1.024	1.02	53.6	44.7	53.0	44.2	1525	1271	3745	3121	48.0	40.0	398.0	331.7	0.4	28.5
UCLA	951792	IC	04/20/06	1.025	2.40	44.0	35.2	25.0	20.0	1813	1450	2335	1868	24.0	19.2	182.0	145.6	0.8	41.2
UCLA	951789	IC	04/21/06	1.028	1.80	52.0	37.1	34.0	24.3	2425	1732	3018	2156	37.0	26.4	139.0	99.3	0.8	46.6
UCLA	951787	IC	04/22/06	1.025	1.50	56.0	44.8	43.0	34.4	1781	1425	2308	1846	60.0	48.0	353.0	282.4	0.8	31.8
UCLA	951790	IC	04/23/06	1.025	1.40	68.0	54.4	56.0	44.8	845	676	924	739	28.0	22.4	161.0	128.8	0.9	12.4
Paris	995462	IC	07/03/06	1.025	2.80	12.0	9.6	4.0	3.2	686	549	992	794	17.0	13.6	89.0	71.2	0.7	57.2
Paris	994203	IC	07/11/06	1.022	1.30	17.0	15.5	15.0	13.6	1210	1100	989	899	20.0	18.2	80.0	72.7	1.2	71.2
Paris	994277	IC	07/13/06	1.029	2.50	24.0	16.6	11.0	7.6	1842	1270	1321	911	55.0	37.9	100.0	69.0	1.4	76.8
Paris	994276	IC	07/14/06	1.026	1.50	21.0	16.2	17.0	13.1	1594	1226	2041	1570	85.0	65.4	333.0	256.2	0.8	75.9
Paris	994075	IC	07/15/06	1.022	1.80	22.0	20.0	13.0	11.8	1017	925	823	748	27.0	24.5	71.0	64.5	1.2	46.2
Paris	995474	IC	07/20/06																
	Screen			1.026	4.90	60.0	46.2	14.0	10.8	230	177	215	165	96.0	73.8	224.0	172.3	1.1	3.8
	A Confirm			1.026	11.40	61.4	47.2	5.2	4.0										
	A Screen (Repeat)			1.026	5.10	49.0	37.7	11.0	8.5	1658		1639		97.0		227.0		1.0	33.8
	B Confirm			1.026	11.00	61.7	47.5	5.7	4.4										
Paris	994080	IC	07/22/06	1.023	2.50	23.0	20.0	11.0	9.6	919	799	771	670	39.0	33.9	94.0	81.7	1.2	40.0
Paris	994171	IC	07/23/06	1.026	1.00	11.0	8.5	12.0	9.2	802	617	758	583	17.0	13.1	51.0	39.2	1.1	72.9
UCLA	1501850	OOC	08/03/06	1.006	1.50	8.0	26.7	5.0	16.7	532	1773	1284	4280	7.0	23.3	88.0	293.3	0.4	66.5
UCLA	497104	OOC	08/21/06	1.023	0.90	73.0	63.5	83.0	72.2	5444	4734	10163	8837	271.0	235.7	870.0	756.5	0.5	74.6

* Analysis of the sample was distorted due to the presence of an inhibition of the derivatization step.

TAB 30

URINE RESULTS FROM WADA ACCREDITED LABS

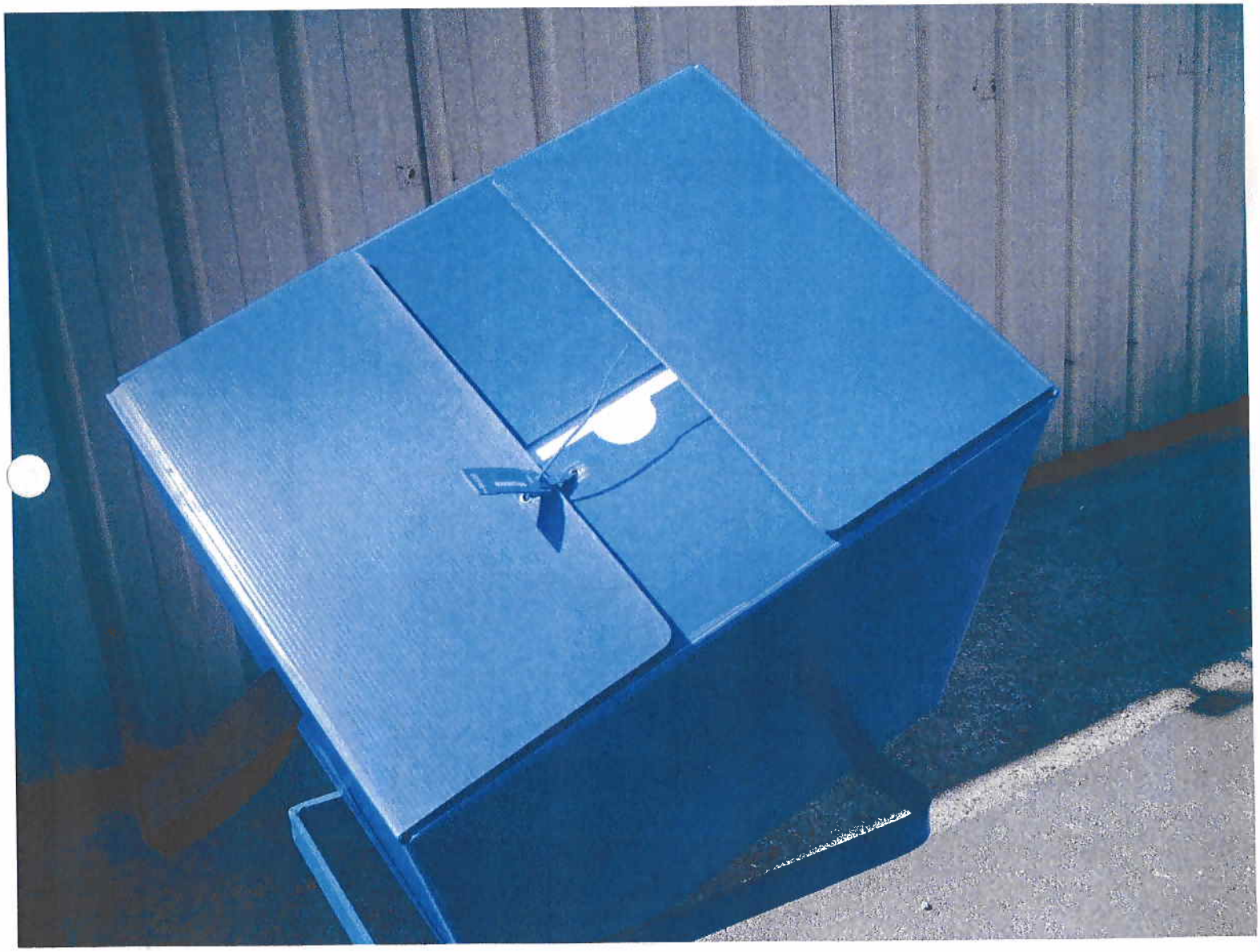
Floyd Landis

Lab	Bottle#	Type	Collection	SG	TE	[Test]	[Test]cr	[Epi]	[Epi]cr	[Andr]	[Andro]cr	[Etio]	[Etio]cr	[5aAD]	[5aAD]cr	[5bAD]	[5bAD]cr	Andro/Etio	Andro/T
Paris	176593		06/16/02	1.023	1.3	28.0	24.3	21.0	18.3	1502	1306	1515	1317	27.0	23.5	184.0	160.0	1.0	53.6
UCLA	466193	OOC	12/08/02	1.020	1.1	16.1	16.1	15.0	15.0	1692	1692	1676	1676	45.0	45.0	169.0	169.0	1.0	105.1
Paris	808345		07/26/03	1.018	1.3	22.0	24.4	18.0	20.0	848	942	1267	1408	18.0	20.0	109.0	121.1	0.7	38.5
Madrid	336873		08/15/03	1.007	1.5	84.1	240.3	57.3	163.7	4111	11746	2951	8431	73.2	209.1	392.0	1120.0	1.4	48.9
UCLA	476315	OOC	11/26/03	1.023	1.1	14.0	12.2	14.7	12.8	859	747	1633	1420	37.0	32.2	153.0	133.0	0.5	61.4
Lisbon	277821	IC	02/22/04	1.011	1.0	19.6	35.6	18.9	34.4	887	1613	754	1371	18.9	34.4	39.1	71.1	1.2	45.3
Paris	289130	IC	03/09/04	1.018	1.3	22.0	24.4	19.0	21.1	1429	1588	1436	1596	25.0	27.8	88.0	97.8	1.0	65.0
Madrid	342599	IC	09/04/04	1.019	1.7	25.8	27.2	14.5	15.3	1761	1854	1902	2002	27.9	29.4	143.9	151.5	0.9	68.3
Madrid	342856	IC	09/11/04	1.014	2.0	7.5	10.7	3.7	5.3	395	564	438	626	5.8	8.3	18.4	26.3	0.9	52.7
Madrid	342850	IC	09/12/04	1.025	1.4	39.9	31.9	29.4	23.5	2605	2084	2702	2162	32.4	25.9	144.9	115.9	1.0	65.3
Madrid	348369	IC	09/13/04	1.019	1.3	25.5	26.8	19.4	20.4	1646	1733	1301	1369	22.3	23.5	153.1	161.2	1.3	64.5
Madrid	347883	IC	09/14/04	1.019	1.0	36.2	38.1	37.9	39.9	2529	2662	1991	2096	32.7	34.4	288.7	303.9	1.3	69.9
UCLA	485155	OOC	01/08/05	1.012	0.8	17.2	28.7	23.8	39.7	933	1555	1310	2183	30.0	50.0	98.0	163.3	0.7	54.2
UCLA	920460	IC	04/21/05	1.015	1.5	11.0	14.7	7.0	9.3	710	947	697	929	9.0	12.0	32.0	42.7	1.0	64.5
UCLA	920462	IC	04/22/05	1.025	1.2	24.0	19.2	21.0	16.8	1416	1133	1491	1193	30.0	24.0	110.0	88.0	0.9	59.0
Paris	874535*	IC	07/17/05	1.036	2.2	32.0	17.8	15.0	8.3	570	317	576	320	22.0	12.2	41.0	22.8	1.0	17.8
UCLA	491607	OOC	10/07/05	1.026	1.1	40.6	31.2	41.9	32.2	1898	1460	2459	1892	138.0	106.2	435.0	334.6	0.8	46.7
UCLA	493084	OOC	11/05/05	1.018	1.1	29.8	33.1	30.0	33.3	1367	1519	1668	1853	75.0	83.3	255.0	283.3	0.8	45.9
UCLA	493091	OOC	01/06/06	1.025	0.9	29.5	23.6	33.7	27.0	1284	1027	3985	3188	40.0	32.0	253.0	202.4	0.3	43.5
UCLA	951826	IC	02/22/06	1.026	1.8	28.0	21.5	16.0	12.3	1006	774	1148	883	18.0	13.8	86.0	66.2	0.9	35.9
UCLA	951875	IC	02/23/06	1.022	1.5	29.0	26.4	19.0	17.3	1222	1111	1283	1166	20.0	18.2	71.0	64.5	1.0	42.1
UCLA	951828	IC	02/24/06	1.025	1.5	29.0	23.2	20.0	16.0	796	637	1977	1582	26.0	20.8	137.0	109.6	0.4	27.4
UCLA	951866	IC	02/25/06	1.025	1.7	35.0	28.0	22.0	17.6	1621	1297	2079	1663	38.0	30.4	205.0	164.0	0.8	46.3
UCLA	951872	IC	02/26/06	1.016	1.8	20.0	25.0	12.0	15.0	1106	1383	1359	1699	14.0	17.5	85.0	106.3	0.8	55.3
Paris	876013	IC	03/08/06	1.027	1.5	65.0	48.1	46.0	34.1	2302	1705	2755	2041	39.0	28.9	202.0	149.6	0.8	35.4
Paris	874285	IC	03/09/06	1.022	1.5	37.0	33.6	27.0	24.5	1305	1186	1362	1238	26.0	23.6	129.0	117.3	1.0	35.3
Paris	872364	IC	03/10/06	1.022	1.3	39.0	35.5	31.0	28.2	1302	1184	1696	1542	28.0	25.5	167.0	151.8	0.8	33.4
Paris	872357	IC	03/11/06	1.024	1.6	61.0	50.8	41.0	34.2	1905	1588	2509	2091	55.0	45.8	308.0	256.7	0.8	31.2
Paris	876004	IC	03/12/06	1.016	1.6	36.0	45.0	24.0	30.0	1298	1623	1385	1731	28.0	35.0	152.0	190.0	0.9	36.1
UCLA	1504999	OOC	04/10/06	1.024	1.0	53.6	44.7	53.0	44.2	1525	1271	3745	3121	48.0	40.0	398.0	331.7	0.4	28.5
UCLA	951792	IC	04/20/06	1.025	2.4	44.0	35.2	25.0	20.0	1813	1450	2335	1868	24.0	19.2	182.0	145.6	0.8	41.2
UCLA	951789	IC	04/21/06	1.028	1.8	52.0	37.1	34.0	24.3	2425	1732	3018	2156	37.0	26.4	139.0	99.3	0.8	46.6
UCLA	951787	IC	04/22/06	1.025	1.5	56.0	44.8	43.0	34.4	1781	1425	2308	1846	60.0	48.0	353.0	282.4	0.8	31.8
UCLA	951790	IC	04/23/06	1.025	1.4	68.0	54.4	56.0	44.8	845	676	924	739	28.0	22.4	161.0	128.8	0.9	12.4
Paris	995462	IC	07/03/06	1.025	2.8	12.0	9.6	4.0	3.2	686	549	992	794	17.0	13.6	89.0	71.2	0.7	57.2
Paris	994203	IC	07/11/06	1.022	1.3	17.0	15.5	15.0	13.6	1210	1100	989	899	20.0	18.2	80.0	72.7	1.2	71.2
Paris	994277	IC	07/13/06	1.029	2.5	24.0	16.6	11.0	7.6	1842	1270	1321	911	55.0	37.9	100.0	69.0	1.4	76.8
Paris	994276	IC	07/14/06	1.026	1.5	21.0	16.2	17.0	13.1	1594	1226	2041	1570	85.0	65.4	333.0	256.2	0.8	75.9
Paris	994075	IC	07/15/06	1.022	1.8	22.0	20.0	13.0	11.8	1017	925	823	748	27.0	24.5	71.0	64.5	1.2	46.2
Paris	995474	IC	07/20/06																
	Screen			1.026	4.9	60.0	46.2	14.0	10.8	230	177	215	165	96.0	73.8	224.0	172.3	1.1	3.8
	A Confirm			1.026	11.4	61.4	47.2	5.2	4.0										
	A Screen (Repeat)			1.026	5.1	49.0	37.7	11.0	8.5	1658		1639		97.0		227.0		1.0	33.8
	B Confirm			1.026	11.0	61.7	47.5	5.7	4.4										
Paris	994080	IC	07/22/06	1.023	2.5	23.0	20.0	11.0	9.6	919	799	771	670	39.0	33.9	94.0	81.7	1.2	40.0
Paris	994171	IC	07/23/06	1.026	1.0	11.0	8.5	12.0	9.2	802	617	758	583	17.0	13.1	51.0	39.2	1.1	72.9
UCLA	1501850	OOC	08/03/06	1.006	1.5	8.0	26.7	5.0	16.7	532	1773	1284	4280	7.0	23.3	88.0	293.3	0.4	66.5
UCLA	497104	OOC	08/21/06	1.023	0.9	73.0	63.5	83.0	72.2	5444	4734	10163	8837	271.0	235.7	870.0	756.5	0.5	74.6
				Average	1.50														
				STDEV	0.46														

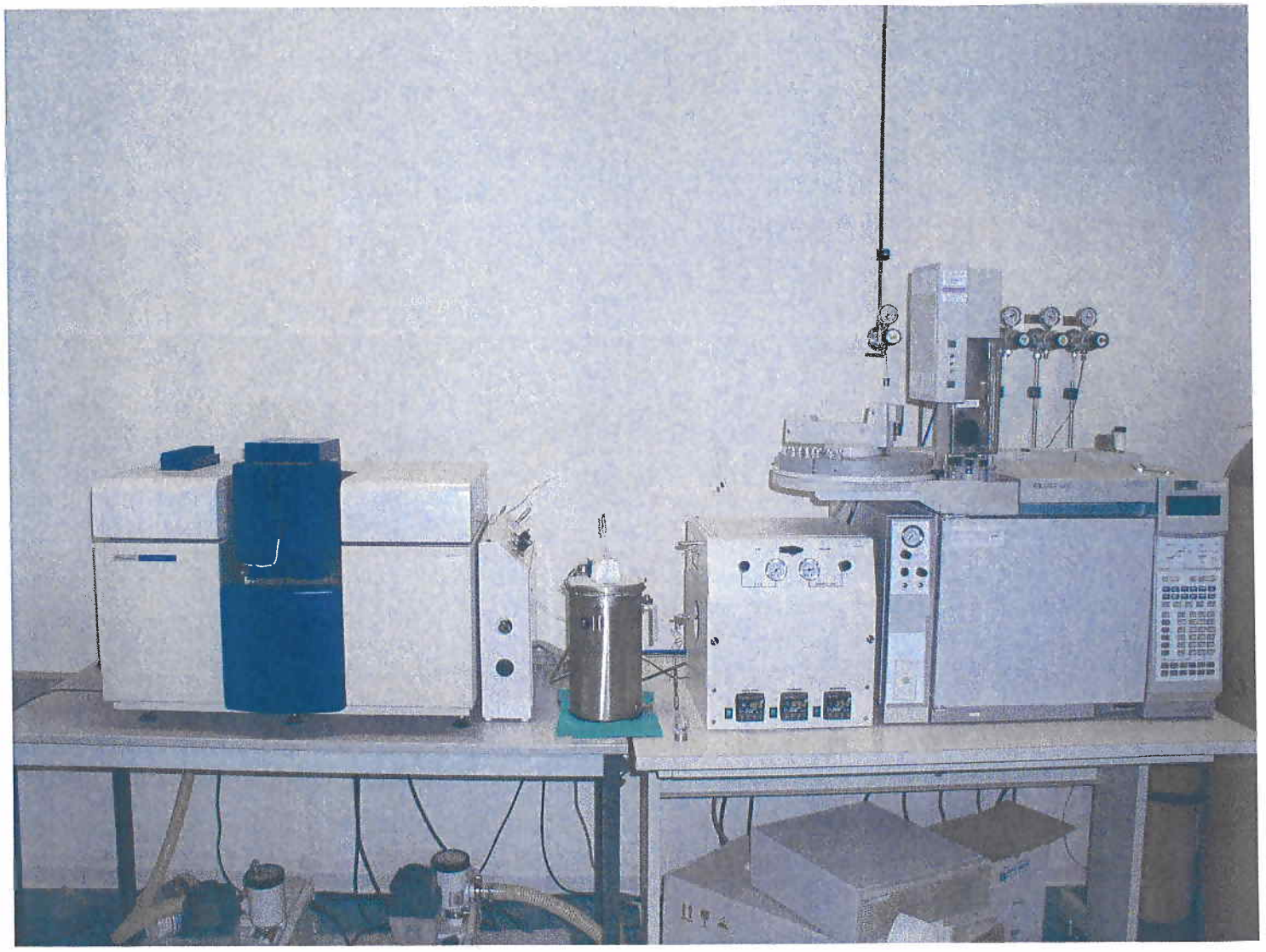
* Analysis of the sample was distorted due to the presence of an inhibition of the derivatization step.

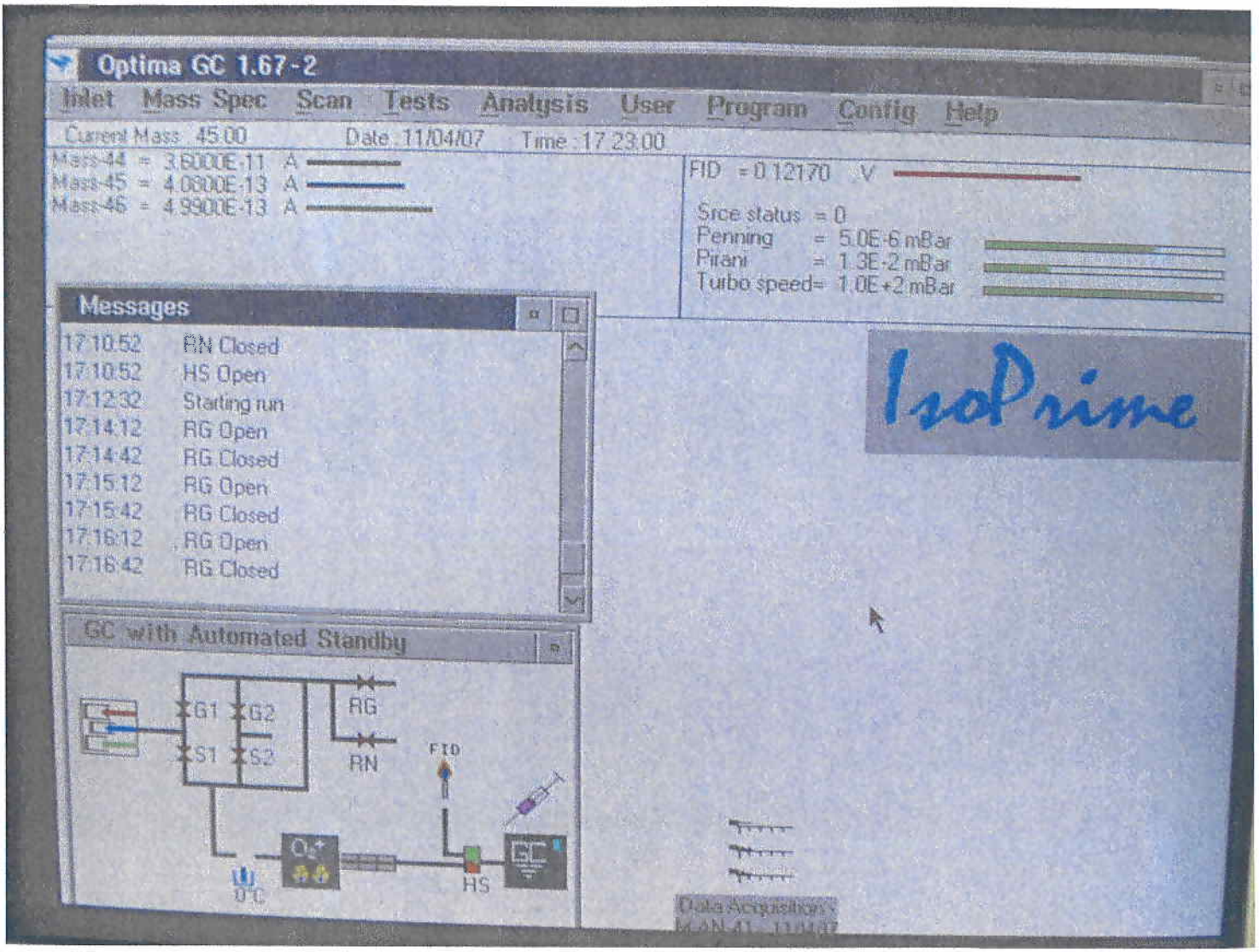
TAB 31

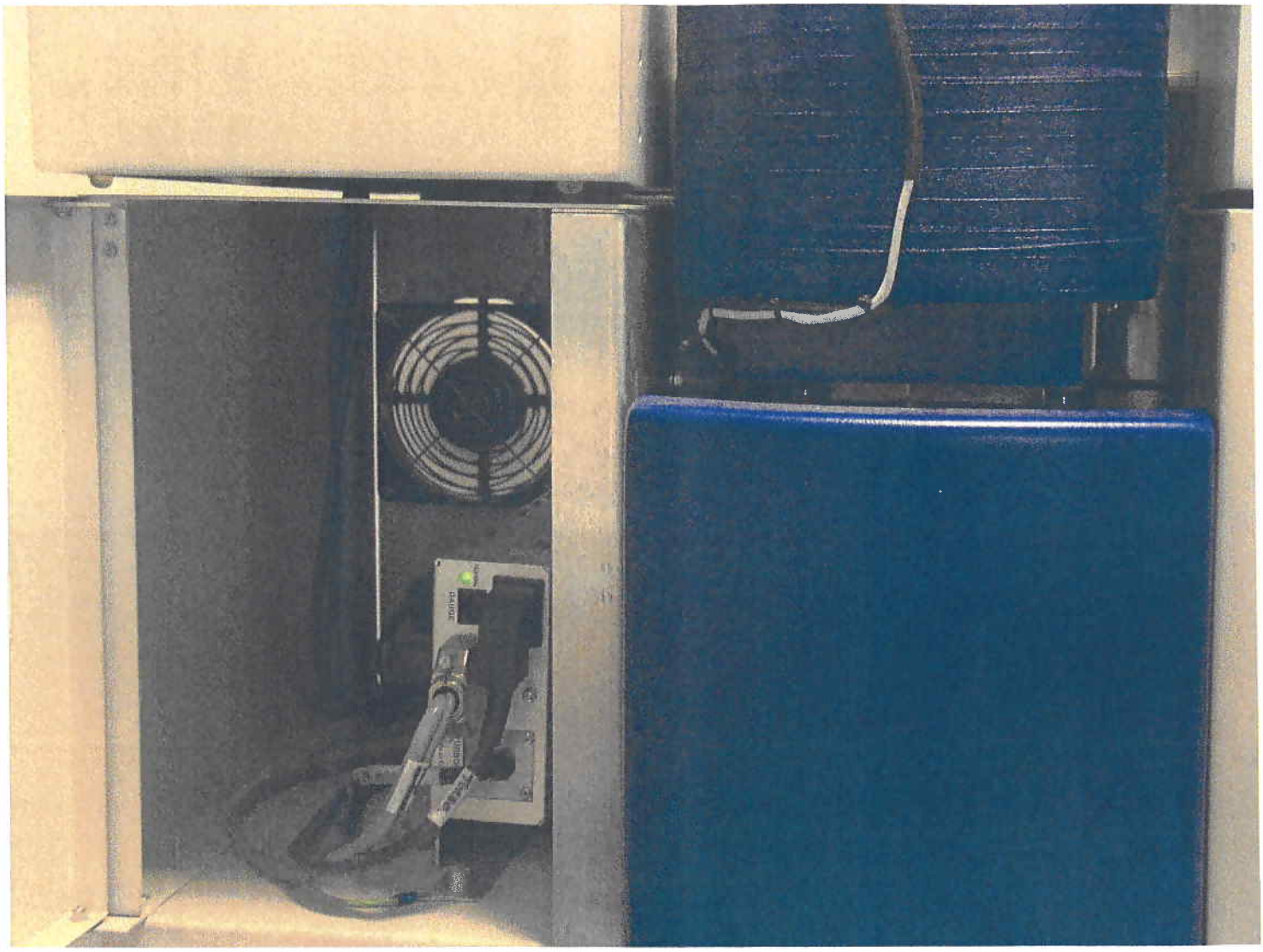
TAB 31



TAB 32




Optima GC 1.67-2
Inlet Mass Spec Scan Tests Analysis User Program Config Help
Current Mass 45.00 Date 11/04/07 Time 17:23:00
Mass-44 = 3.8000E-11 A
Mass-45 = 4.0800E-13 A
Mass-46 = 4.9900E-13 A
FID = 0.12170 V
Srce status = 0
Penning = 5.0E-6 mBar
Pirani = 1.3E-2 mBar
Turbo speed= 1.0E+2 mBar
Messages
17:10:52 RN Closed
17:10:52 HS Open
17:12:32 Starting run
17:14:12 RG Open
17:14:42 RG Closed
17:15:12 RG Open
17:15:42 RG Closed
17:16:12 RG Open
17:16:42 RG Closed
IsoPrime
GC with Automated Standby
G1
G2
S1
S2
RG
RN
FID
HS
0°C



TAB 32 A

Respondent's Exhibit 49 alleged documentation errors concerning IRMS are reproduced below in boldface.

USADA 0120, 1 alleged error, Custody, "No record of overnight storage."
There is no such error. Custody of overnight storage is documented correctly on that very page, USADA 0120. In the column labeled *"Opération"* (Operation), the fifth row labeled *"Troisième extraction"* (Third extraction) shows:

Date (Date): 22/07/06

Heure de début (start time): 19 h 40

Identification du matériel utilisé (Identification of material used): Rapid Trace: RTORD

Date (Date): 23/07/06

Récupéré à (retrieved at): 9 h 05

Paraphe (initials or signature): [signature of analyst Cynthia Mongongu]

In other words, the sample was on the Rapid Trace robot for automated extraction, from 19 h 40 on 22/07/06 to 9 h 05 on 23/07/06. It is a common and widely accepted laboratory practice to sign samples over to laboratory items such as a robot, hood, or analytical instrument such as a GC-MS.

USADA 0149, alleged Sample ID problem, "According to the record, sample f2b was used in Fraction F2. I see no explanation why this was done. There is no record of this subtraction in the chain of custody.
There is no problem here. That the datafile with a name ending with f2b was used for (not "in") fraction F2 is correct. The reason for this is simple: when the sample's Fraction F2 was injected into the GC-MS, the data revealed overload (in other words, the amount of material exceeded the capacity of the system, which departs from optimal conditions, because it is detrimental to good compound separation), therefore the sample's Fraction F2 was diluted and reinjected. The resulting datafile, with a name ending in f2b, gave better data, therefore these are the data that were used.

The chain of custody on p USADA 0121-0122 is correct. The statement, "There is no record of this subtraction in the chain of custody." is irrelevant since there was no "subtraction."

USADA 0257, 2 alleged errors, Custody, "The chain of custody records that the 'B' sample IRMS was performed on August 3, 2006. There is no record of an analysis performed on August 3, 2006. There is no chain of custody documenting the work performed on August 4, 2006."
- The statement, "There is no record of an [IRMS] analysis performed on August 3, 2006." is correct. The B sample IRMS analysis was conducted on August 4, 2006.
- The statement, "The chain of custody records that the 'B' sample IRMS was performed on August 3, 2006." is incorrect because page USADA 0257 is not a chain of custody document, instead it is a chain of custody summary prepared only to assist the reader. The date for the IRMS analysis on page USADA 0257 should have been August 4, 2006.

- The statement, "There is no chain of custody documenting the work performed on August 4, 2006." is incorrect. The chain of custody is documented correctly on page USADA 0302, top table, second row: ISOPRIME 1. It is a common and widely accepted laboratory practice to sign samples over to laboratory items such as an analytical instrument (e.g., IRMS), robot, or hood.

USADA 0300, 1 alleged error, Custody, "No record of overnight storage."
There is no such error. Custody of overnight storage is documented correctly on that very page, USADA 0300. In the column labeled *"Opération"* (Operation), the third row labeled *"Acétylation"* (Third extraction) shows:

Date (Date): 03/08/06

Heure de début (start time): 18 h 05

Identification du matériel utilisé (Identification of material used):
DLU* Anhydride acétique 9.11.08
DLU* Pyridine 10/2006

Date (Date): 04/08/06

Récupéré à (retrieved at): 9 h 14

Paraphe (initials or signature): [identification code 26 and initials of analyst Claire Frelat]

In other words, the sample was on undergoing acetylation in a fume hood, from 18 h 05 on 03/08/06 to 9 h 14 on 04/08/06. It is a common and widely accepted laboratory practice to sign samples over to laboratory items such as a hood, robot, or analytical instrument.

In addition, before leaving the lab room at the end of the day on August 3, 2006, the analyst lowered the hood glass door and used lab tape to tape across the opening to warn other lab staff not to open it. The analyst also taped a warning note outside the lab room door. These are all common and widely accepted practices in a lab with restricted access.

USADA 0309, 1 alleged error, Custody, "No record of this work, and following pages in this section, in chain of custody."

There is no such error and this statement is incorrect. First of all, chain of custody is documented only for the sample, not for Mix Acetate, which is what page USADA 0309 shows. Second, the following pages in this section, USADA 0309-0322 show data from the pre-IRMS compound identification by GC-MS analysis on MSD22 (MDS22 as seen on page USADA 0313, line 1 and 3). The chain of custody is documented correctly on page USADA 0301, top table, second row, "MSD22." It is a common and widely accepted laboratory practice to sign samples over to laboratory items such as an analytical instrument (e.g., GC-MS), robot, or hood.

USADA 0323, 1 alleged error, Sample ID, "According to the record, sample f3 was used in Fraction F2. There was a similar problem in the 'A' sample, USADA 149. The corresponding trace graphs are too small to be legible. Analyzing the wrong aliquot fraction cannot be ruled out.

There is no such error. On summary pages USADA 0323-0324, in one of three places where the sample Fraction 2 datafile name 17807474**f2** should appear, 17807474**f3** appears instead (top table on page USADA 0323). However, every bit of the data attributed to file 17807474**f2** as it was recopied onto the summary pages did come from the raw data from file 17807474**f2** seen on page USADA 0317: note that the first line on that page is Data File Name 17807474f2.

The statement that "Analyzing the wrong aliquot fraction cannot be ruled out" is incorrect. If Fraction F3 had been injected into the GC-MS instead of Fraction F2, the data would not show the presence of andro and etio because they are found only in Fraction F2.

Fractions in which andro and etio are present, therefore the corresponding graphs have two big peaks dwarfing the noise: Blank Urine F2 (page USADA 0316), sample F2 (page USADA 0318).

Fractions in which andro and etio are absent, therefore the corresponding graphs have no big peaks, only noise: Blank urine F1 (page USADA 0312), sample F1 (USADA 0314), Blank Urine F3 (page USADA 0320), sample F3 (page USADA 0322).

The statement, there was a similar problem in the 'A' sample, is incorrect. There was no problem with the A sample.

USADA 0352, 2 alleged errors, Calculation, "The report erroneously reports androsterone as (positive) 3.51 rather than (the negative) -3.51." and "The conclusion states that two metabolites were abnormal. Androsterone is wrongly reported as abnormal, its value is not beyond the WADA's minimum positivity criteria (3.00‰) plus error range (0.8‰). This is clearly explained in Spirito.[1]

[1] Spirito, E. et al. The role of measurement uncertainty in doping analysis. Int. J. Risk Assessment and Management. 5 (2/3/4), 374-386, (2005).

Respondent's uncertainty argument is addressed starting at paragraph 90 of USADA's brief.

TAB 32 B

LNDD	ENREGISTREMENT	Codification : E-CE-01 Version : C Date : 05/12/2005 1/2

Formulaire d'ouverture de l'échantillon B

= Form opening of sample B

Demande faite par WADA / UCI Le(s) : 31/07/2006

Date : 03/08/2006

Identification des personnes présentes : legal representative UCI on WADA representative

	Laboratoire		Témoin indépendant	Expert	Autre
Nom	Mongongu	Feelay	[illegible]	D. de Boer	CAROLINE HATTON MARTIAL SAUGY UCI EXPERT
Signature					

Destockage du flacon A : Date 03/08/06 Heure 9 h 12 Opérateur : 18

Destockage du flacon B : Date 03/08/06 Heure 9 h 12 Opérateur : 18

Lieu de déstockage : Chambre froide n° 5

Conditions de stockage du flacon B : -80°C ☐ -20°C ☒ +4°C ☐

Système : Versapak ☐ Berlinger ☒ Autre ☐ N° B995474

Nature du milieu biologique : Urine

Identification : Conformité par rapport au procès verbal de contrôle antidopage : Oui ☒ Non ☐

Intégrité des scellés : Oui ☒ Non ☐

=seals intact Yes

Validation (Signatures) : legal representatives. UCI on WADA representative

Laboratoire	Témoin indépendant	Expert	Autre

LNDD	ENREGISTREMENT	Codification : E-CE-01 Version : C Date : 05/12/2005 2/2
Formulaire d'ouverture de l'échantillon B = Form: opening of sample B		

Ouverture : = opening:

N° du flacon : B 995436

Volume : 2,5 mL (approximativement)

(si le milieu est congelé attendre sa décongélation pour mesurer le volume)

Validation (Signatures) :

Laboratoire	Témoin indépendant	Expert	AFLD

Cet enregistrement est à archivé dans le dossier analytique de la contre expertise.

LNDD | ENREGISTREMENT = FORM | Codification: E-TE-03A | Version: M | Date: 13/09/2005

ALIQUOT WORK-UP SHEET FOR B CONFIRMATION BY GC

FICHE DE SUIVI DES ALIQUOTES POUR LA CONFIRMATION CONTRE EXPERTISE EN GC

Echantillon: 178/07 - 395474 (nombre maximum T/E par page USADA 0072) — Mode opératoire d'extraction : M-GK-06B

Date	Appareil	Température en °C	Valeur affichée	Paraphe
03/08/06	pHmet n° 7	21,8	5,18	
03/08/06	Refract n° 2		1,025	

Date de mise à l'ambiant de l'échantillon : 01/08/06 — Heure de mise à l'ambiant : 9H12

Prise d'essai PE = aliquot : 2 mL — Heure de la PE : 11H05 — Paraphe :

	Donneur	Densité	Facteur de dilution	Vol (en mL)	Vol eau ajouté (en mL)
Echantillon			1/1	2 ml	
Blanc	45	1,022	1/1	2 ml	

Substance (TP, REF, SI ...)	Code sol ref	Conc sol ref	Volume prélevé en µL 1	2	3	4	5	6	Conc ref dans PE en ng/ml 1	2	3	4	5	6
SI : méthyltestostérone	S13-0467	4 ng/µL	50						100					
Testostérone	H10-035-2	1 mg/µl	6						2					
Epitestostérone	H3-032.1.1	1 mg/µl	4						2					
Testostérone	H10-034-1	10 mg/µl	6						30					
Epitestostérone	H3-032.1.1	1 mg/µl	10						5					
Testostérone	H10-035	100 mg/µl	36						180					
Epitestostérone	H3-032.2	10 mg/µl	6						30					
Testostérone	H10-034	100 mg/µl	72						360					
Epitestostérone	H3-033.2	10 mg/µl	12						60					

Opération	Date	Heure début	Récupéré à	Identification du matériel utilisé	Paraphe
Lavage	03/08/06	11H45	12H05		
Evaporation	03/08/06	12H30	13H00	Bain à sec n° : à froid	
Incubation	—	—	—	T (°C) : —	
Mise à pH	03/08/06	13H05		Code tampon : T130706.07	
Hydrolyse	03/08/06	13H10	14H10	Dlu enzyme : 01/08/06. Etuve n° 5	23
Extraction	03/08/06	14H20	15H25	Dlu NH4OH : RT Gilson n° 1	
Stockage	—	—	—	Lieu : —	
Evaporation	03/08/06	15H30	16H00	Bain à sec n° 17	
Dérivation	03/08/06	16H05	16H25	Dérivation 1 — Bain à sec n° 13 — Micro-ondes — Code ou dlu du réactif 1 : 15/08/06 — Dérivation 2 — Bain à sec n° — Dlu réactif 2	
Evaporation				Bain à sec n° :	
Reprise					
Stockage				Lieu :	

dlu : date limite d'utilisation

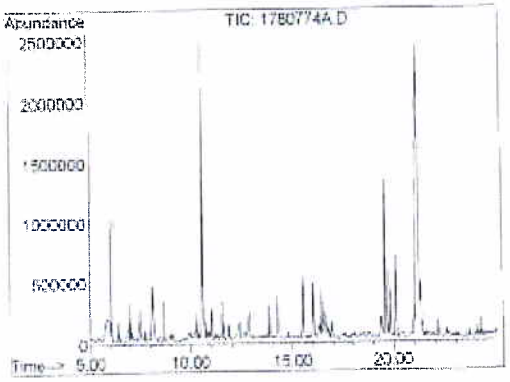
Cet enregistrement est à archiver dans le dossier de confirmation

```
File:           D:\MSD20\AOUT06\0308\1780774A.D
Operator:       23
Date Acquired:  2 Aug 2006  19:43
Instrument:     MSD 20
Method File:    MAN27
Sample Name:    B 178/07 995474 TE
Misc Info:
Vial Number   : 4
```

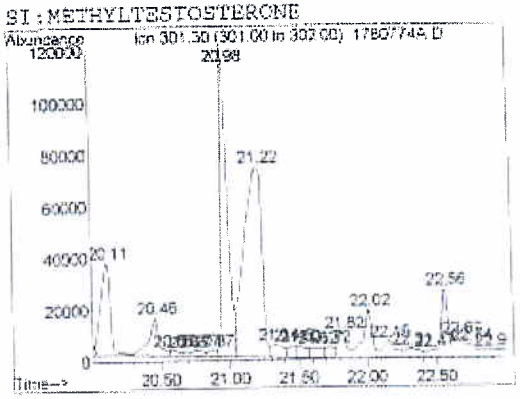
filename found on page USADA 0288

1 of 3 sample vials

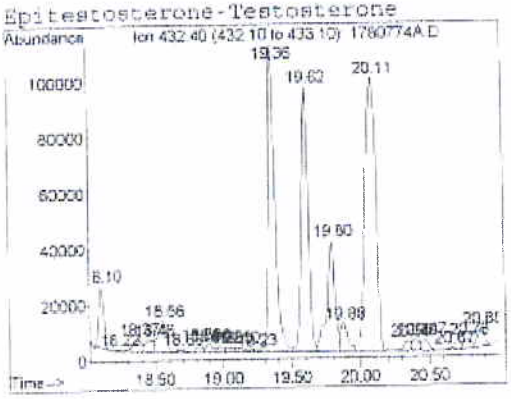
Analyse quantitative:Testosterone/Epitestosterone



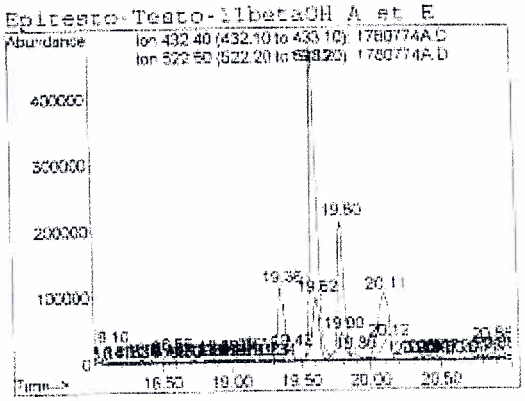
SI:METHYLTESTOSTERONE



Epitestosterone-Testosterone



Epitesto-Testo-1betaOH A et E



Data File Path	D:\MSD20\AOUT06\0308\
Data File Name	1780774A.D
Operator	23
Date Acquired	8/3/2006 16:43
Acq. Method File	MAN27
Sample Name	B 178/07-985474 TE
Vial Number	4
Calibration Title	Quantification du rapport T/E
Last Calibration Update	Fri Aug 04 07:32:46 2006

filename found on page USADA 0288

#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	20.96	301.3	Methyltestosterone	3971127	100.00	ng/mL
2)		18.59	432.4	Epitestosterone	342595	5.94	ng/mL
3)	*	19.36	432.4	Testosterone	3733052	63.15	ng/mL

areas found on page USADA 0288

Calcul du rapport T/E = Calculation of T/E ratio

Area Surface	Concentration
10.9	10.6

T/E ratio found on page USADA 0288

D:\MSDchem\Custrpt\MAN27.CRT

8/4/2006 7:39 AM

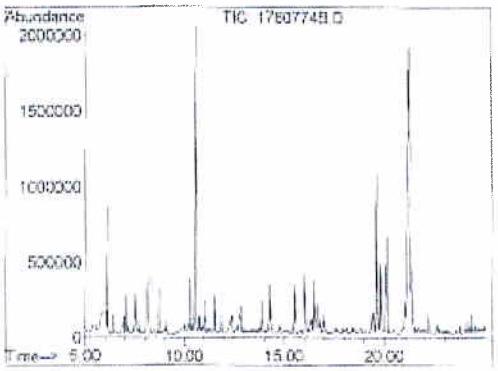
USADA 0278

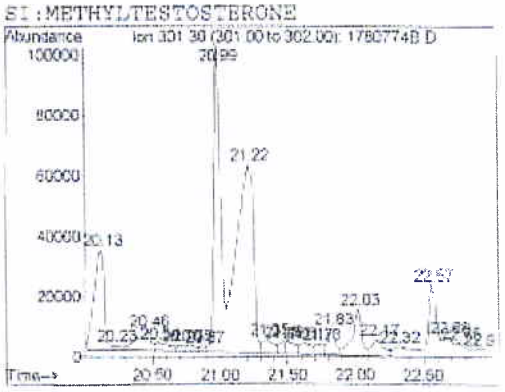
```
File:           D:\Msd20\acqu06\0308\1780774B.D
Operator:       23
Date Acquired:  3 Aug 2006  19:14
Instrument:     MSD 20
Method File:    MAN27
Sample Name:    B 178/07 995474 TE
Misc Info:
Vial Number   : 5
```

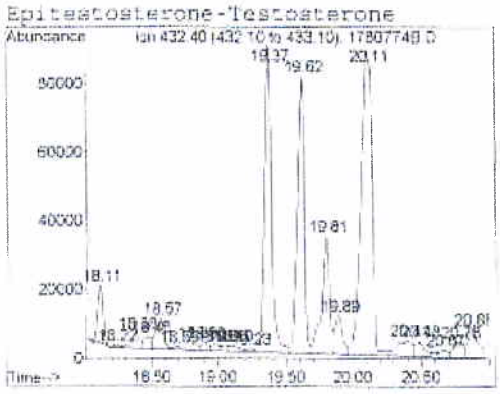
filename found on page USADA 0288

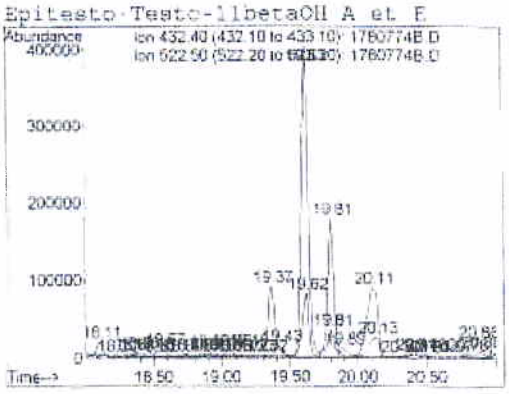
2 of 3 sample vials

Analyse quantitative:Testosterone/Epitestosterone









D:\MSD20\AOUT06\0308\1780774B.D

Data File Path	D:\MSD20\AOUT06\0308\
Data File Name	1780774B.D
Operator	23
Date Acquired	8/3/2006 19:14
Acq. Method File	MAN27
Sample Name	B 178/07 695474 TE
Vial Number	5
Calibration Title	Quantification du rapport T/E
Last Calibration Update	Fri Aug 04 07:32:46 2006

filename found on page USADA 0288

#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	20.99	301.3	Methyltestosterone	3356149	100.00	ng/mL
2)		18.57	432.4	Epitestosterone	279871	5.75	ng/mL
3)	*	19.36	432.4	Testosterone	3079122	61.64	ng/mL

areas found on page USADA 0288

Calcul du rapport T/E = Calculation of the T/E ratio

Area Surface	Concentration
11.0	**10.7**

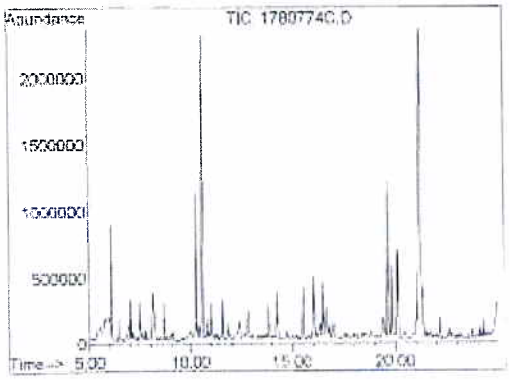
T/E ratio found on page USADA0288

File: D:\Med20\acqu06\0308\1780774C.D
Operator: ZS
Date Acquired: 3 Aug 2006 19:45
Instrument: MSD 20
Method File: MAN27
Sample Name: B 178/07 995474 TE
Misc Info:
Vial Number : 6

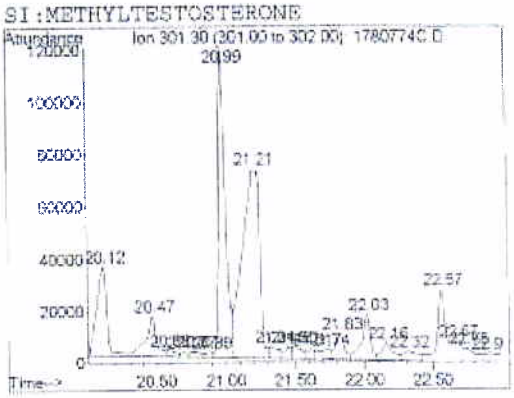
filename found on page USADA 0288

3 of 3 sample vials

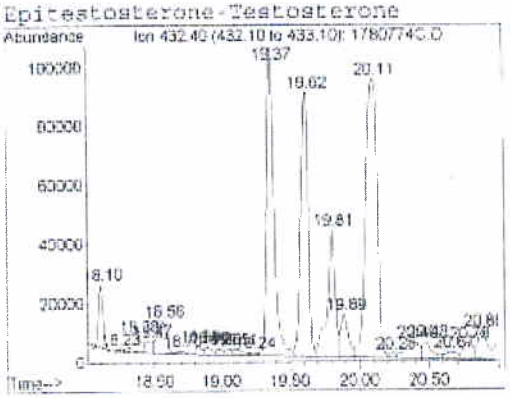
Analyse quantitative:Testosterone/Epitestosterone



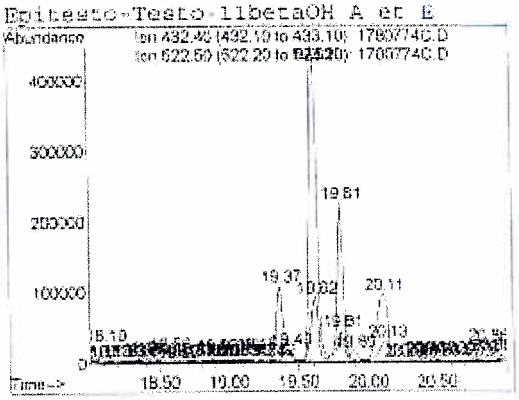
SI:METHYLTESTOSTERONE



Epitestosterone-Testosterone



Epitesto-Testo-libetaOH A et E



Data File Path	D:\MSD20\AOUT06\0308\
Data File Name	1780774C.D
Operator	23
Date Acquired	8/3/2006 19:45
Acq. Method File	MAN27
Sample Name	B 178/07995474 TE
Vial Number	6
Calibration Title	Quantification du rapport T/E
Last Calibration Update	Fri Aug 04 07:32:46 2006

filename found on page USADA 0288

#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	20.99	301.3	Methyltestosterone	4098783	100.00	ng/mL
2)		18.56	432.4	Epitestosterone	329392	5.55	ng/mL
3)	*	19.37	432.4	Testosterone	3670090	60.18	ng/mL

areas found on page USADA 0288

Calcul du rapport T/E = Calculation of the T/E ratio

Area Surface	Concentration
11.1	10.8

T/E ratio found on page USADA 0288

LNDD	ENREGISTREMENT	Code : E-FCR-04B2 Version : A Date : 03/08/2006 Page : 1/1

FICHE D'ANALYSE / RESULTAT - CONFIRMATION SEMI-QUANTITATIVE T/E (trois aliquotes)

Paraphe :

Echantillon : 478/07 994474 Dilution : 1/ 1

Ion de quantification Testo et Epitesto : 432 Ion de quantification SI : 301

Concentration de la référence 1 : Testosterone: 30 Epitestosterone : 5 T/E théorique : 6

Concentration de la référence 2 : Testostérone: 180 Epitestosterone : 30 T/E théorique : 6

Concentration de la référence 3 : Testostérone: 360 Epitestosterone : 60 T/E théorique : 6

Fichier		Surface du SI	Surface Testosterone	Surface Epitestosterone
REF1	REF1TE	3782021	1845917	294592
REF2	REF2TE	3011193	7860237	1324358
REF3	REF3TE	3783290	20557109	3442296
1780774A		3971127	3733052	342595
1780774B		3356149	3079122	279871
1780774C		4098783	3670090	329392

RESULTAT DE L'ECHANTILLON

T/E area ratio

Fichier	Concentration Testosterone		Concentration Epitestostérone		Rapport T/E en surface
1780774A	63,2	ng/mL	5,9	ng/mL	10,9
1780774B	61,6	ng/mL	5,8	ng/mL	11,0
1780774C	60,2	ng/mL	5,6	ng/mL	11,1
mean Moyenne	61,7	ng/mL	5,7	ng/mL	11,0
Ecart type	1,5	ng/mL	0,2	ng/mL	0,1
Valeur finale	61,7	ng/mL	5,7	ng/mL	

Partie à remplir par le responsable

Seuil de déclaration du rapport T/E (en surface) : 4

Incertitude (liée à la méthode) pour le rapport T/E : 30% pour l'Epitestosterone : 30% pour la Testosterone : 20%

Valeur basse du rapport T/E : 7.7 Résultat : Anormal : X

Valeur haute du rapport T/E : 14.3 Inclassable :

Négatif :

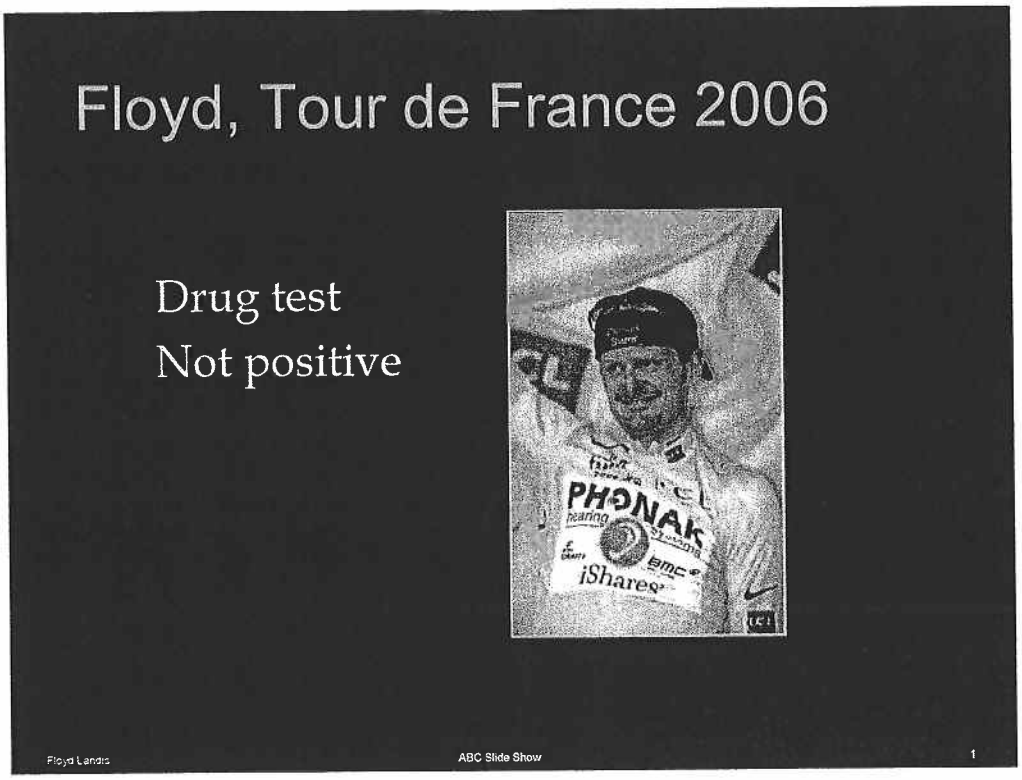
Correction des concentrations en Testosterone et Epitestosterone par la densité (cf doc E-INC-03) :

		PARAPHE
Densité affichée	1.025	
Numero du réfractomètre	9	
Densité corrigée :	1.025	
Facteur de correction	0.74	
Concentration corrigée de Testosterone	4.57 ng/mL	
Concentration corrigée d'Epitestosterone	4.2 ng/mL	

Ecart ± :

Cet enregistrement est à archiver dans le dossier de confirmation

TAB 33



Prepared by Arnie Baker, MD.

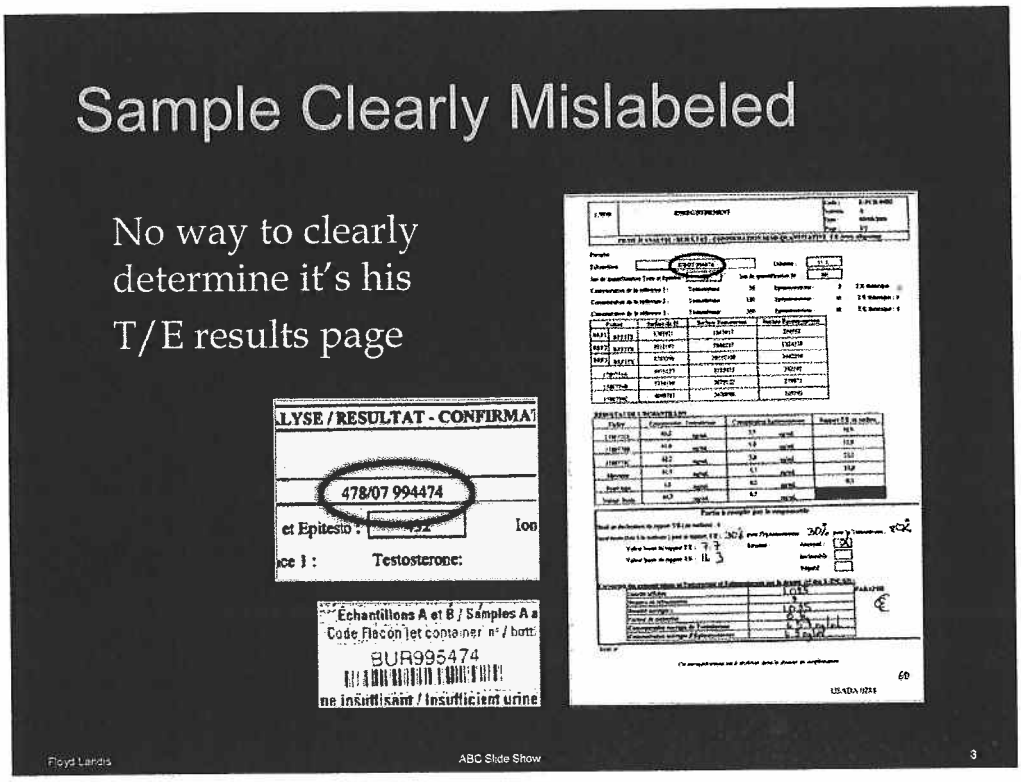
Baker is a retired San Diego physician. He is a long-time Landis coach and advisor.

While in active medical practice, Baker had over a decade experience in medical peer review and quality assurance.

Baker has written about bicycling medicine for the lay public, International Olympic Committee, and medical community.

Arnie Baker

Digitally signed by Arnie Baker
DN: cn=Arnie Baker, c=US, email=arnie@arniebakercycling.com
Date: 2006.10.11 13:47:24 -07'00'



The lab made many errors in its analysis.

One of the easiest examples of lab error to see involves sample identification or labeling.

This is the page summarizing the testosterone and epitestosterone results from the A sample.

The sample number has two parts: The lab identification number and the athlete's identification number.

The lab identification number is wrong.

The lab identification number is 178/07—not 478/07.

The athlete's identification number is wrong. 994474 is not Floyd's number.

Floyd's number is the number in the barcode label taken from his attestation page. It is 995474.

TAB 34

Institute of Biochemistry
German Sport University Cologne
WADA-accredited Laboratory for Doping Analysis

WADA research project

Progress Report

March 2007

Schänzer, W., Geyer, H., Mareck, U., Platen, P.*, Flenker, U., Piper, T., Schmechel, A., Lüdke, S., Schrader, Y., Thevis, M.

The detection of the misuse of testosterone gel

Institute of Biochemistry, German Sport University Cologne, Germany
* Institute of Cardiology and Sports Medicine, Department of Cellular and Molecular Sports Medicine, German Sport University Cologne, Germany

Table of contents

1 Introduction 1

2 Methods 1

3 Results and Discussion 2

3.1 Blood samples 2

3.2 Steroid profile parameters 7

3.2.1 Discriminating parameters 7

3.2.2 The discriminating power of individual reference ranges 8

3.2.3 Risk of misinterpretation of data after application of T-gel 12

3.3 GC/C/IRMS results 13

4 Conclusions 16

5 Next steps 16

6 References 17

7 Annex 18

1 Introduction

According to the WADA-list of prohibited substances, the use of testosterone is prohibited in sports. In 2000 a new testosterone preparation, a testosterone gel (T-gel), was approved by the Food and Drug Administration of the USA for the testosterone replacement therapy in men for conditions associated with low testosterone. Since 2003 T-gel is also admitted to the European market. Several investigations have shown, that the application of T-gel leads to performance enhancing physiological effects like significant increase of the lean body mass, muscle strength and haemoglobin concentration (1).

Results of a pilot study with hypogonadal men have shown that it is difficult to detect the misuse of T-gel with the actual available methods of doping control (2).

The aim of the study is to develop a method to prove the misuse of testosterone gel i.e the search for a parameter or several parameters with the strongest discriminating power. These parameters may be steroid profile parameters, pituitary gland hormones, carbon isotope ratios and hydrogen isotope ratios of steroids or combinations of these parameters.

2 Methods

In the first part of the project excretion studies with testosterone gel were performed with 18 healthy male volunteers (P1 – P18) between 20 and 30 years. The 18 volunteers were also participants in the WADA approved research project "Endocrine study on the effects of testosterone gel application in male athletes" directed by Prof. Petra Platen. The treatment with testosterone gel was approved by the Local Ethical Committee and the German Federal Institution of Drug Control and Drug Research (BfARM).

Application of testosterone gel

Group 1 (9 healthy male volunteers: P2, P3, P4, P5, P6, P10, P12, P16, P17): For six weeks daily transdermal application of 2 x 5 g Testogel® (Jenapharm, Jena Germany) corresponding to 100 mg testosterone. Application in the morning after the shower according to the recommendation.

Group 2 (9 healthy male volunteers: P1, P7, P8, P9, P11, P13, P14, P15, P18): For six weeks intermittent application of testosterone gel. One week daily transdermal application of 2 x 5 g Testogel® (Jenapharm, Jena Germany) corresponding to 100 mg testosterone. One week without testosterone gel etc. Application in the morning after the shower according to the recommendation.

Collection of urine samples (total number: 1300)

Urine samples were collected

- before the application of testosterone gel: each urine sample for five days
- during the application of testosterone gel: on the 6th day of each week collection of all urine samples
- after the application of testosterone gel: on the 6th and 15th day after the cessation of the application collection of all urine samples

Collection of blood samples

Blood samples were collected (one sample of 3 ml in the morning each)

- before the application of testosterone gel
- during the application of testosterone gel: on the 6th day of each week
- after the application of testosterone gel: on the 6th and 15th day after the cessation of the application

Analyses of the urine samples

All 1300 samples were analysed with GC/MS for endogenous steroid-glucuronides according to the previously described methods (3, 4). Concentrations and ratios were calculated for the following endogenous steroids: androsterone (And), etiocholanolone (Etio), testosterone (T), epitestosterone (E), 5α-androstane-3α,17ß-diol (Adiol) and 5ß-androstane-3α,17ß-diol (Bdiol). Additionally specific gravity and pH were measured.

Selected urine samples of 2 volunteers were analysed also with GC/C/IRMS. As target compounds were chosen the glucuronides of androsterone, etiocholanolone, testosterone and 5α-androstane-3α,17ß-diol and 5ß-androstane-3α,17ß-diol. For androsterone and etiocholanolone the GC/C/IRMS analyses were performed according to the standard operating procedures of the Cologne laboratory (5, 6). For the analyses of testosterone and 5α-androstane-3α,17ß-diol and 5ß-androstane-3α,17ß-diol new purification methods have been developed. The purification method for testosterone is presented in annex 1.

Analyses of blood samples

The blood samples were analysed with standardized commercial electro chemiluminescence immunoassays (Roche Diagnostics) for total testosterone, free testosterone and LH.

3 Results and Discussion

3.1 Blood samples

The results of the analyses of the blood samples are presented in the Figures 1-8.
After continuous application of T-gel the serum testosterone levels exceeded the upper limit of the normal range in 4 volunteers (P4, P6, P10, P12; see Fig.1). For the volunteers P2, P3 and P5 increases of the serum testosterone concentrations, but not above the limit of the normal range, could be observed. The volunteers P16 and P17 showed no change of the serum testosterone levels (Fig. 2). During the intermittent application 5 volunteers reached serum testosterone values above the normal range (Fig. 3), 3 volunteers showed an increase within the normal range and one volunteer showed no change (Fig. 4).
The serum LH-concentrations decreased in 5 volunteers after continuous application of T-gel below the lower limit of the normal range (Fig. 5), 2 volunteers showed a suppression within the normal range and 2 volunteers showed no change (Fig. 6). After intermittent application of T-gel the LH concentrations decreased during application in 6 volunteers (P7, P8, P11, P13, P14, P15). In 3 volunteers no clear decrease could be observed (P1, P9, P18) (see Fig. 7 and 8).

These results show that testosterone has penetrated through the skin into the circulation and has caused physiological effects. There is no clear explanation, why 3 volunteers showed no change of the serum testosterone concentrations.

Fig. 1: Total serum testosterone concentrations [nmol/l] of 4 volunteers before, during and after a 6 week continuous application of testosterone gel (red bars: values during application). Normal range: 12-40 nmol/l

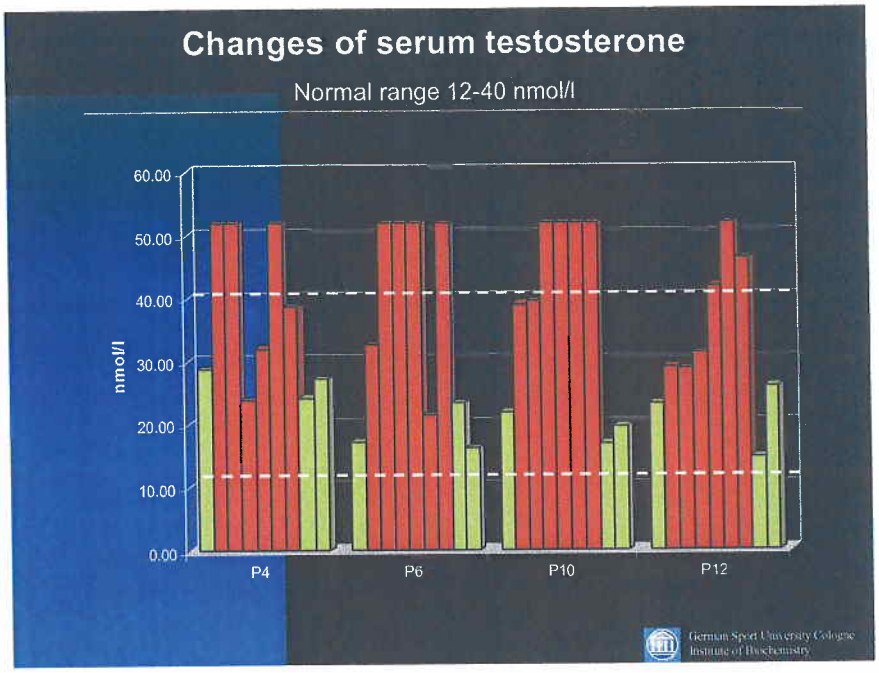


Fig. 2: Total serum testosterone concentrations [nmol/l] of 5 volunteers before, during and after a 6 week continuous application of testosterone gel (red bars: values during application). Normal range: 12-40 nmol/l

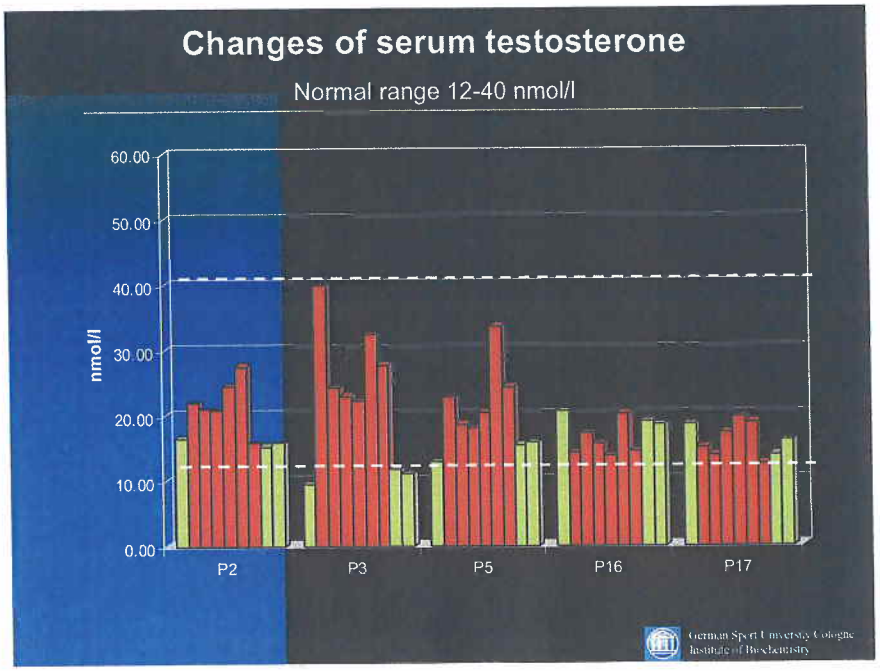


Fig. 3: Total serum testosterone concentrations [nmol/l] of 5 volunteers before, during and after a 6 week intermittent application of testosterone gel (red bars: values during application). Normal range: 12-40 nmol/l

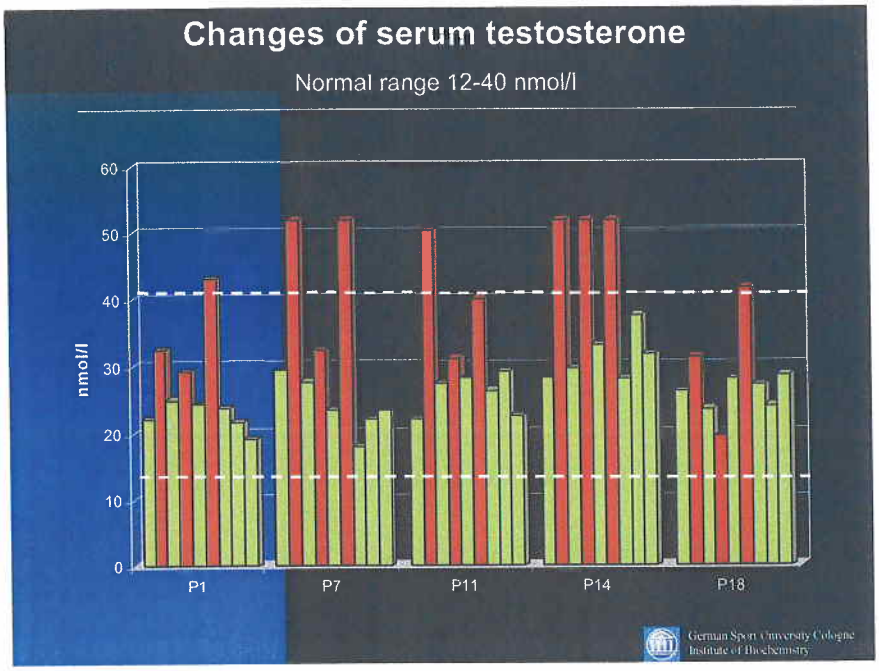


Fig. 4: Total serum testosterone concentrations [nmol/l] of 4 volunteers before, during and after a 6 week intermittent application of testosterone gel (red bars: values during application). Normal range: 12-40 nmol/l

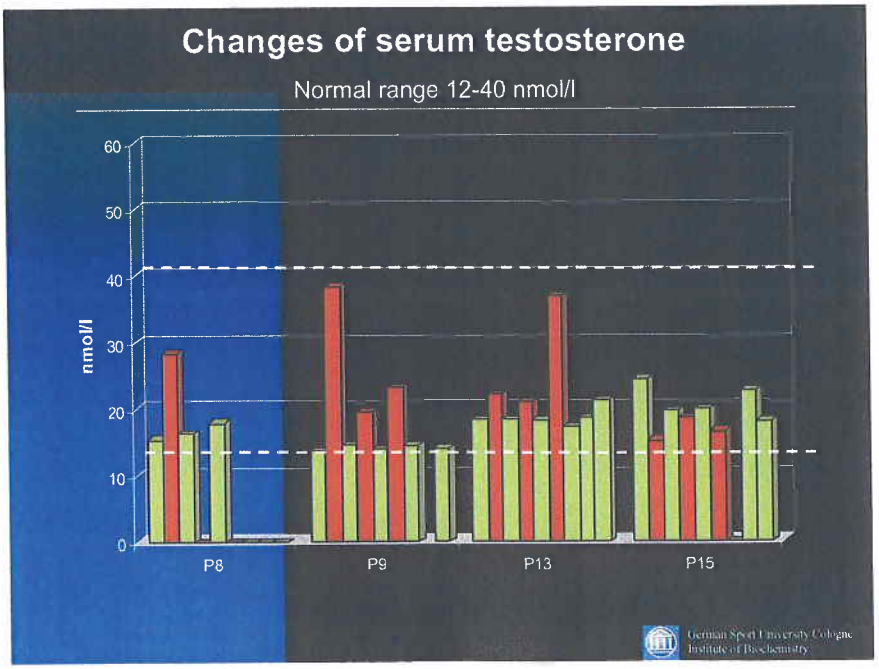


Fig. 5: Serum LH concentrations [IU/l] of 5 volunteers before, during and after a 6 week continuous application of testosterone gel (red bars: values during application). Normal range: 1.7-8.6 IU/l

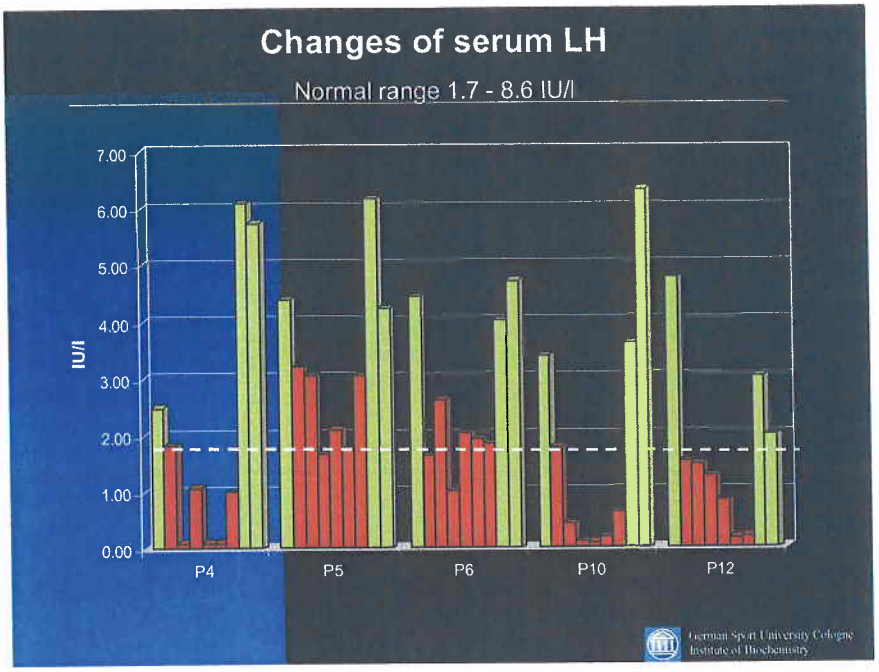


Fig. 6: Serum LH concentrations [IU/l] of 4 volunteers before, during and after a 6 week continuous application of testosterone gel (red bars: values during application). Normal range: 1.7-8.6 IU/l

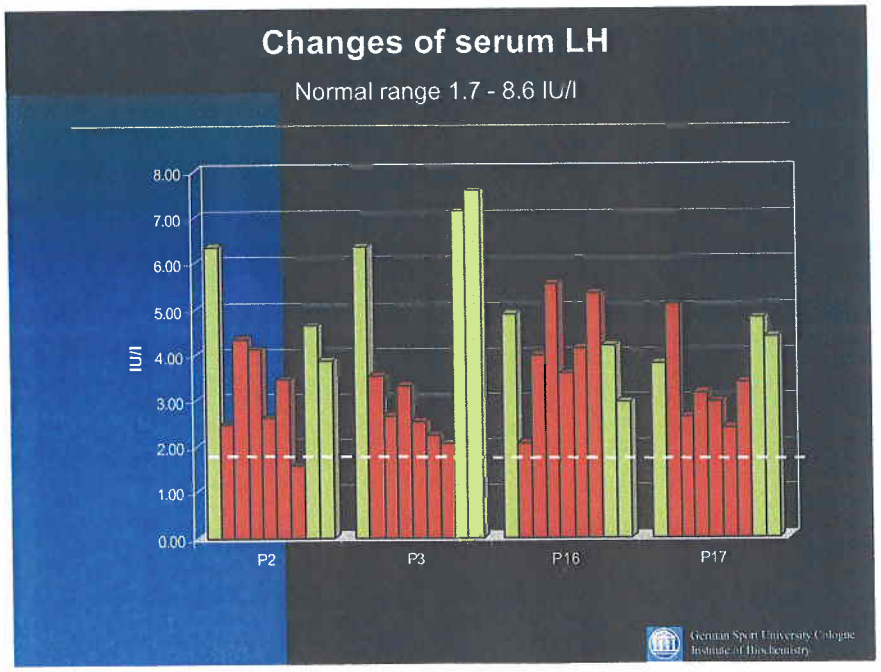


Fig. 7: Serum LH concentrations [IU/l] of 4 volunteers before, during and after a 6 week intermittent application of testosterone gel (red bars: values during application). Normal range: 1.7-8.6 IU/l

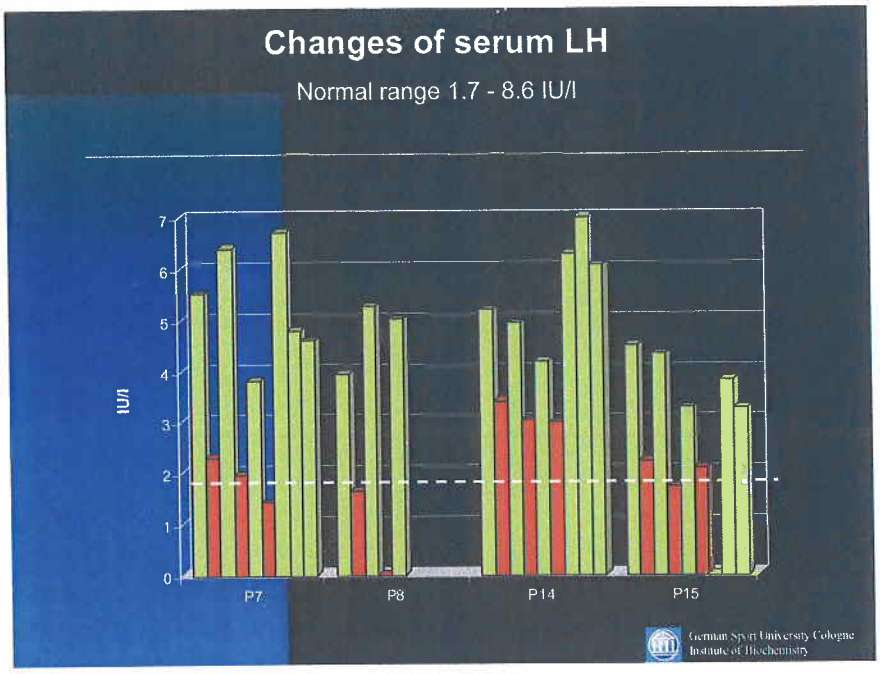
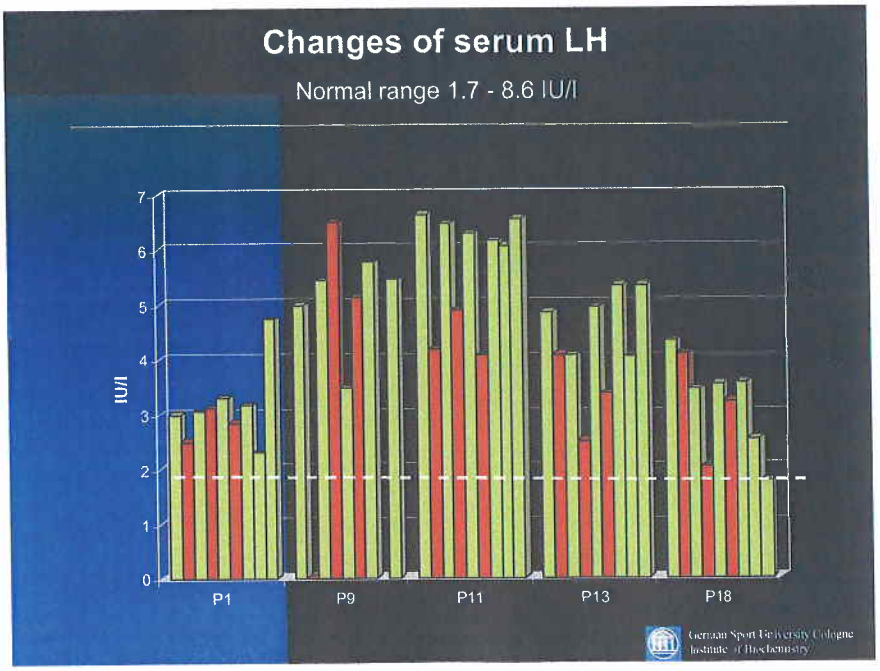


Fig. 8: Serum LH concentrations [IU/l] of 5 volunteers before, during and after a 6 week intermittent application of testosterone gel (red bars: values during application). Normal range: 1.7-8.6 IU/l



3.2 Steroid profile parameters

In annex 2, boxplots of selected steroid profile parameters and scatter diagrams of the T/E ratios of the 18 volunteers are presented.

3.2.1 Discriminating parameters

The most obvious changes of the steroid profile after application of testosterone gel were the changes of the ratios Adiol/E, And/E and T/E. These changes were based on the decrease of the E concentrations and the increase of the T, And and Adiol concentrations. This is shown as an example for volunteer P2 in Figure 9. These results in young healthy volunteers confirm the observations obtained from middle-aged hypogonadal men (2). The results also indicate that after transdermal application the conversion of testosterone to Adiol is much stronger than the conversion to Bdiol. This is shown in Figure 10. After a daily administration of T-Gel for one week the ratio Adiol/Bdiol of volunteer P10 increased for about 150-200%. In another study with another volunteer the daily oral administration of testosterone (1 capsule Andriol/day = 25 mg testosterone) for one week led to an increase of the ratio Adiol/Bdiol of only 30-40% (7). The conversion of testosterone to 5-alpha-steroids after transdermal application is probably due to a high 5-alpha-reductase activity of the skin

Fig. 9: Changes of steroid profile parameters before during and after administration of T-Gel (volunteer P2)

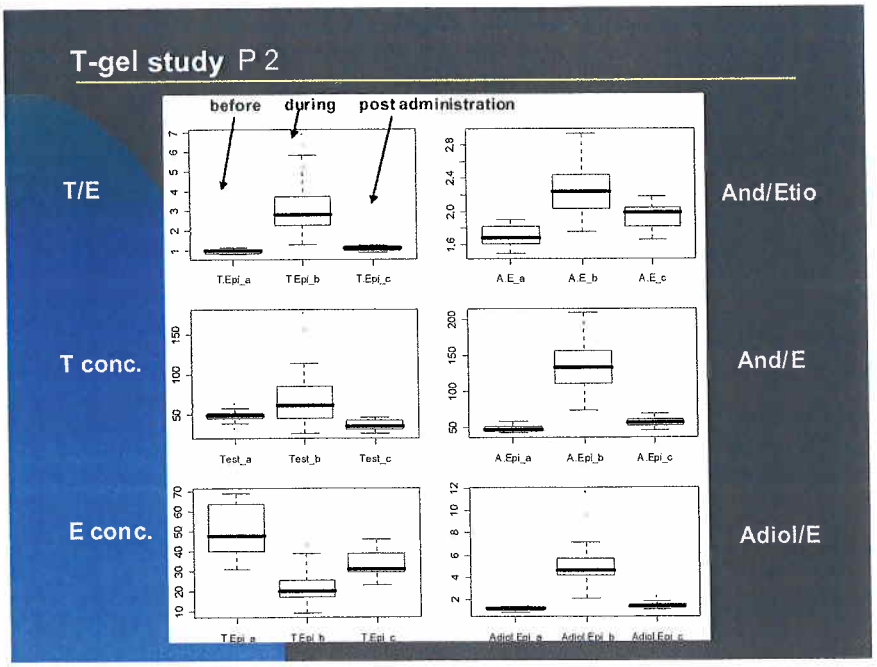
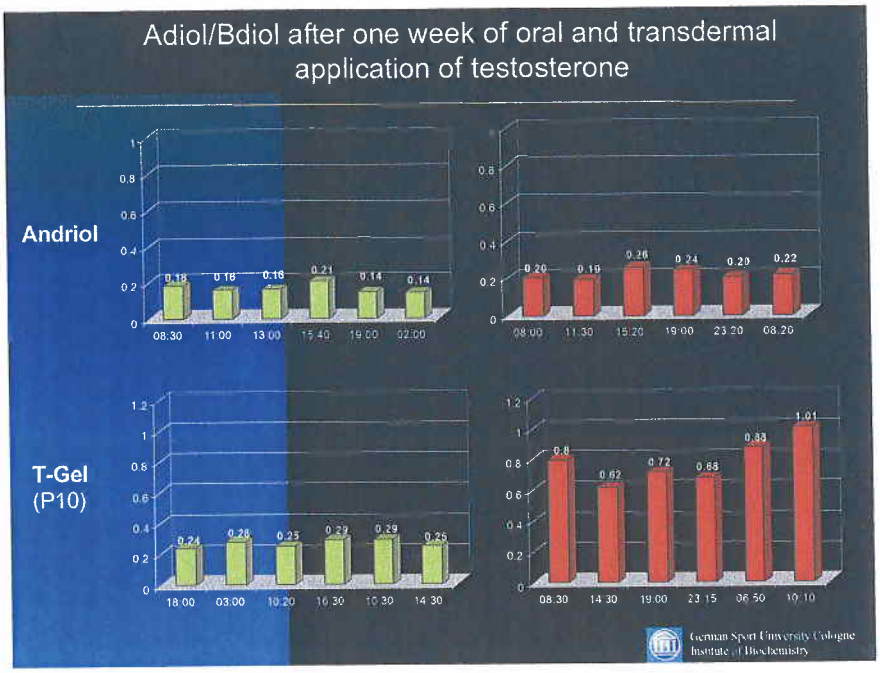


Fig. 10: Adiol/Bdiol ratios during a day after one week of oral (1 tablet Andriol/day = 25 mg testosterone) and transdermal administration of testosterone



3.2.2 *The discriminating power of individual reference ranges*

To compare the discriminating power of the limits of individual reference ranges versus limits of population based reference ranges, the T/E ratio was chosen as parameter. The individual reference range for a volunteer was calculated from the T/E values obtained before the administration of T-Gel. The limits of the individual reference range were defined as mean of the pretest values (about 20-40 values) ± 3 x standard deviation. This is shown in figure 11 for volunteer P6. As limit of the population based reference range was chosen the WADA cut off limit T/E = 4.

As presented in Figure 12 and 13 nearly all samples of volunteers P3 and P4 reach T/E ratios both above the cut off limit of 4 and far above the upper limit of the individual reference ranges. This was not the case for volunteer P5. During the T-Gel application more than 50 % of the T/E ratios didn`t exceed the cut off limit of 4, but nearly all T/E ratios exceeded the upper limit of the individual reference range of 1.86 (Fig. 14). Also individual reference ranges of other ratios, especially the ratios Adiol/E and And/E, show a strong discriminating power (Fig. 15). In Figure 16 are presented data of volunteer P11, who performed an intermittent application of T-Gel. The mean of his pretest T/E ratios was 0.44. During the T-Gel application all T/E-values were far below the cut-off limit of 4, but all values were above the upper limit of the individual reference range of 0.79. These results show that it is necessary to implement individual reference ranges for the detection of the misuse of T-Gel and that the cut off limit of 4 is not sufficient for this purpose. In Figure 16 the other steroid profile parameters of volunteer P11 are shown. Once again the discriminating power of the ratios Adiol/E and And/E is obvious.

Fig. 11: Calculation of the limits of the individual reference range for the T/E ratio for volunteer P6 from the pretest values

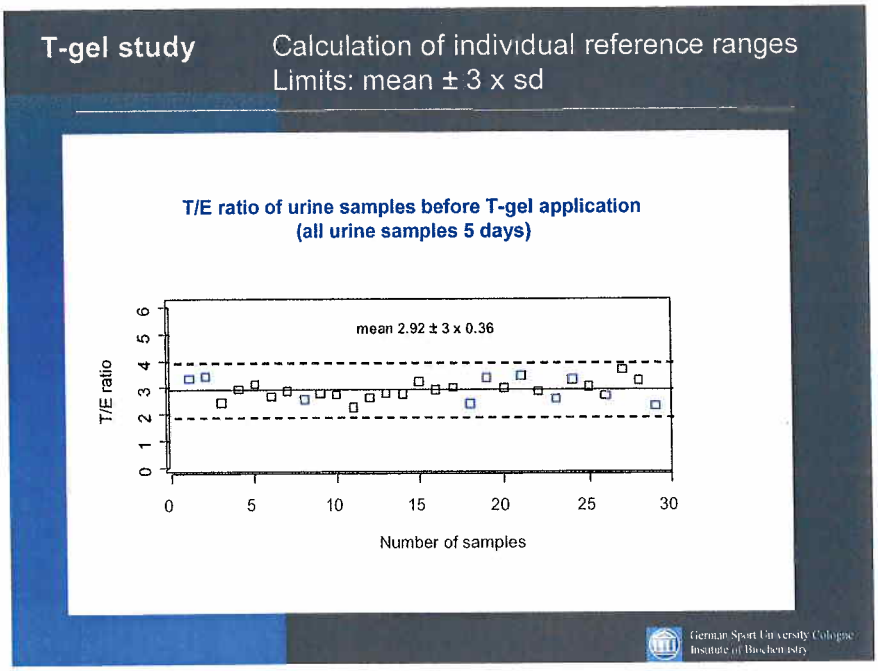


Fig. 12: T/E ratios of volunteer P3 before and during the application of T-Gel. red line: T/E=4; black line: upper limit of individual reference range

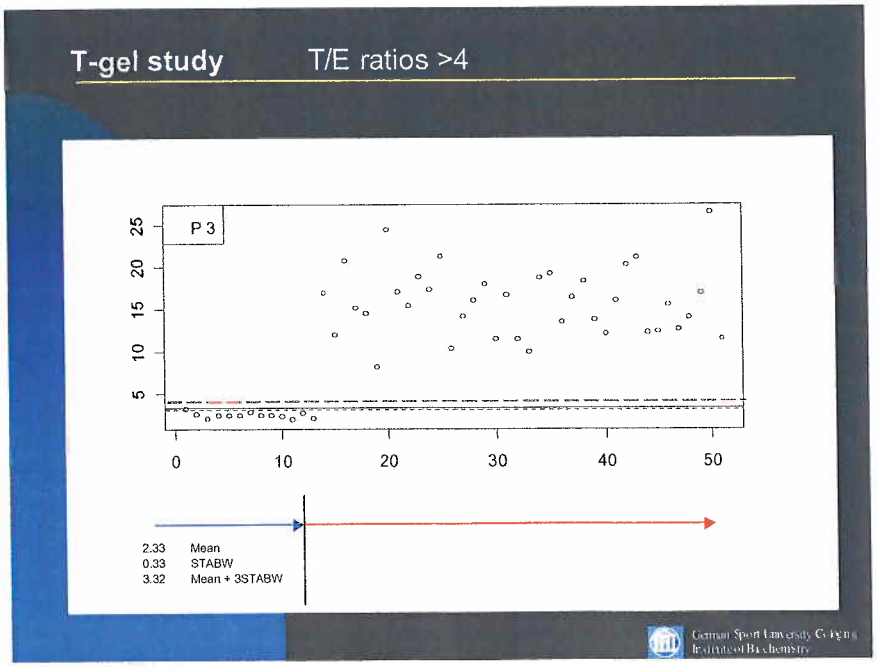


Fig. 13: T/E ratios of volunteer P4 before and during the application of T-Gel. red line: T/E=4; black line: upper limit of individual reference range

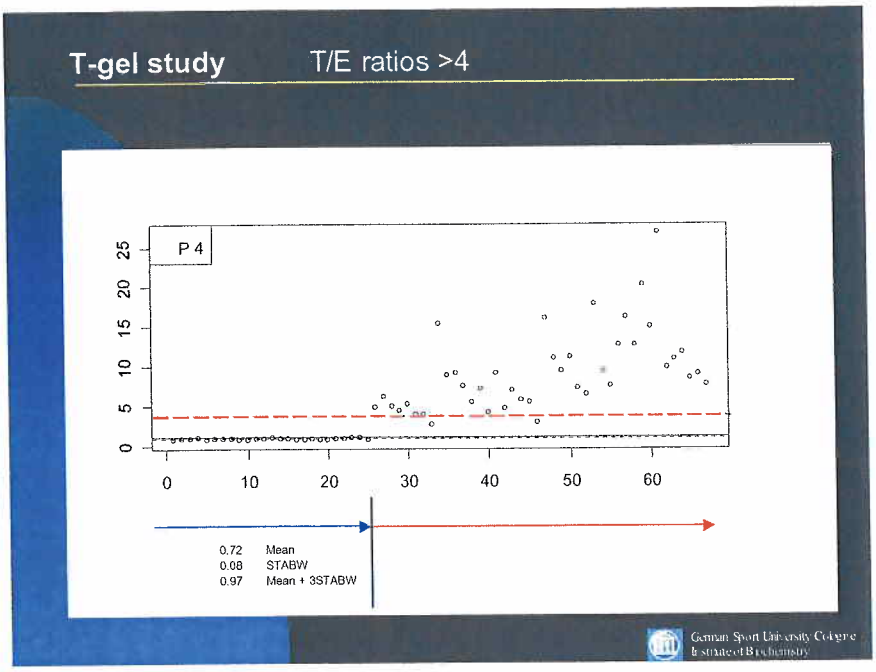


Fig. 14: T/E ratios of volunteer P5 before and during the application of T-Gel. red line: T/E=4; black line: upper limit of individual reference range

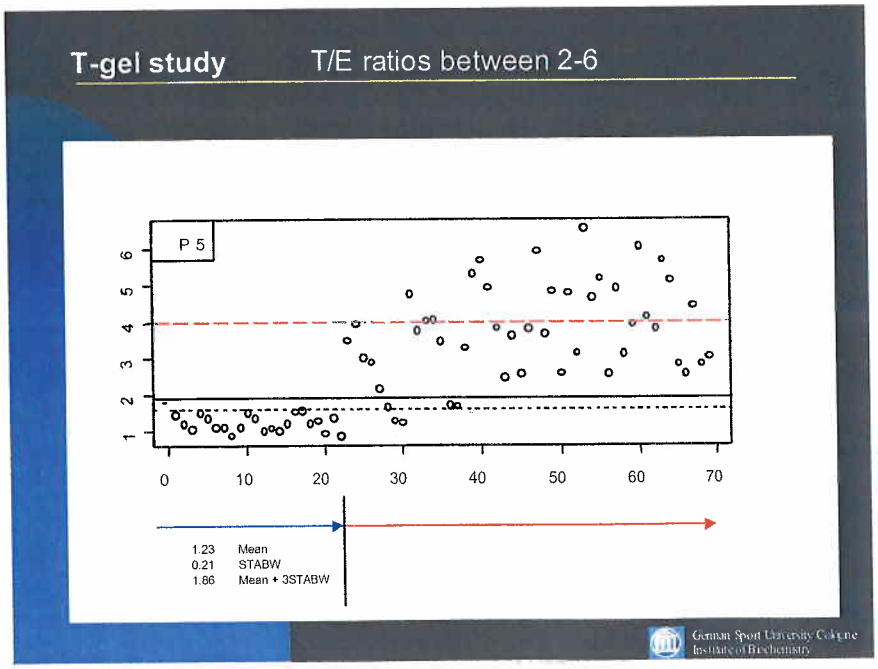


Fig. 15: Changes of steroid profile parameters before during and after administration of T-Gel (volunteer P5)

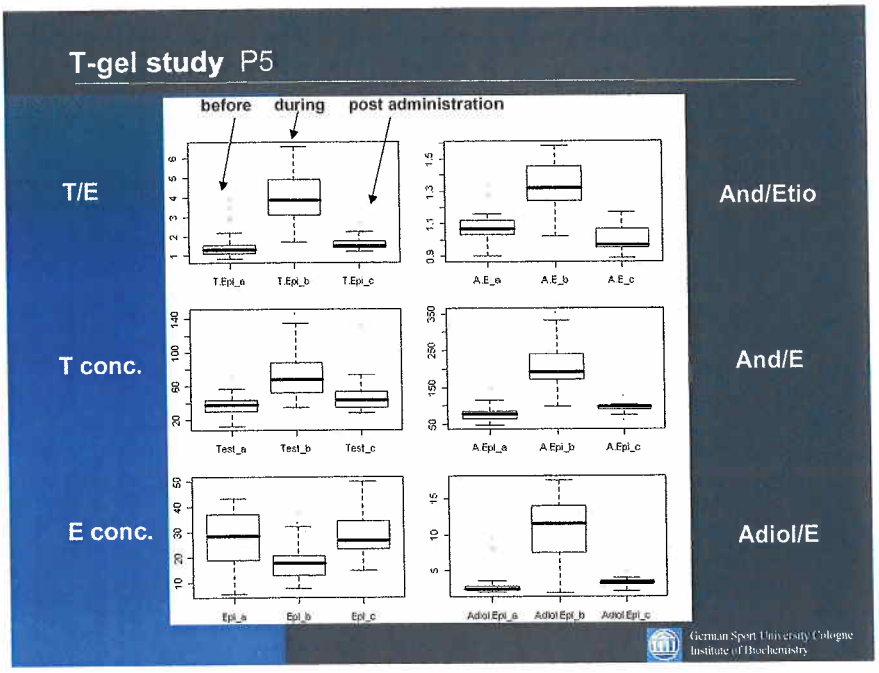


Fig. 16: T/E ratios of volunteer P1 before and during an intermittent application of T-Gel, black line: upper limit of individual reference range

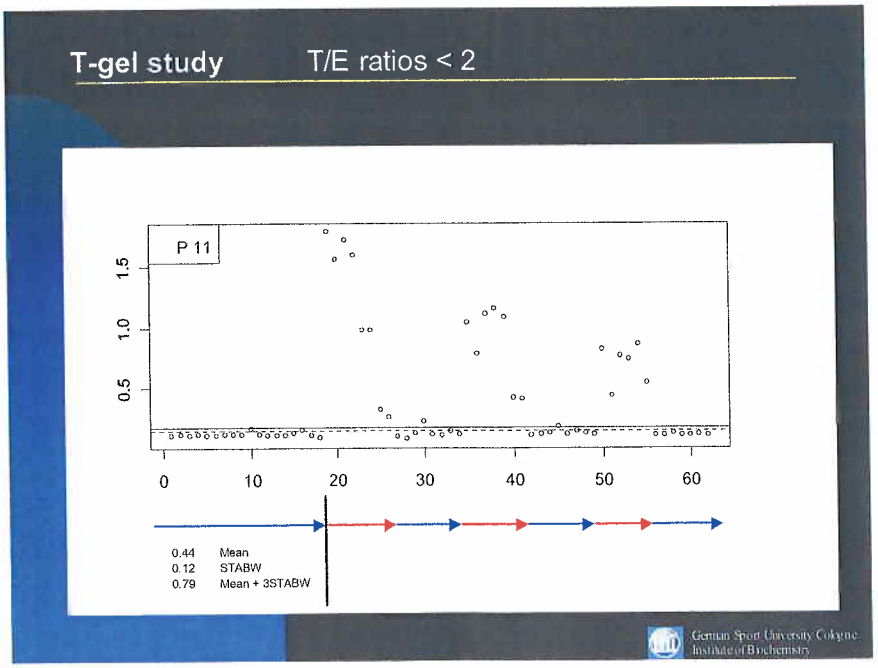
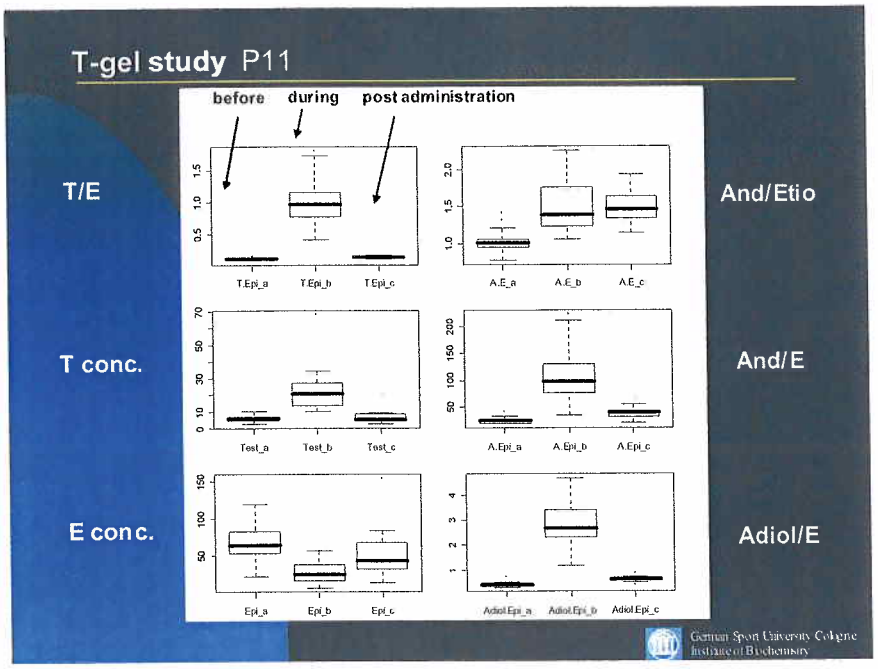


Fig. 17: Changes of steroid profile parameters before during and after administration of T-Gel (volunteer P11)



3.2.3 *Risk of misinterpretation of data after application of T-gel*

The application of T-gel led to constantly increased serum T levels (see Fig. 1-4) and to constantly increased urinary T/E ratios (see Fig. 12, 13, 14, 16 and scatter diagrams in the annex 2) during the application. These results are in accordance with published data from investigations with hypogonadal men (1, 2). This relative "stability" of the T/E ratios after T-gel application is totally different to the strong variations observed after oral or intramuscular application of testosterone (2). The constantly increased T/E ratios after T-gel application may lead to misinterpretations of longitudinal studies i.e. to false negative results. This is shown for volunteer P4.

In Table 1 steroidprofile parameters of volunteer P4 from different days during the testosterone gel application are presented. After 2 weeks of daily T-gel application this volunteer showed on 14th of November 2006 a T/E ratio 8.8. Presuming that this volunteer is an athlete, further data of this subject have to be collected to decide whether this T/E ratio is a natural increased ratio or caused by an application of exogenous steroids (8). The urine samples collected on the 15th and 29th of November and on the 5th of December 2006 corresponding to 2, 4 and 5 weeks respectively of daily application of T-gel showed T/E ratios of 7.1, 6.4 and 7.5 respectively. According to the WADA technical document TD2004EAAS (8) from these data the mean value and the upper limit of the individual reference range, defined as mean + 30% for males, should be calculated. Based on these data the "suspicious" T/E ratio of 8.8 lies within the accepted normal variation of the T/E ratio (Tab. 1) and also the other steroid profile parameters are within the population based reference ranges of male athletes (9) and vary in a normal range. The interpretation of these data of volunteer P4 would be: "Naturally increased T/E ratio based on a low E excretion".

Code	sample coll.	s.g.	And	Etio	E	T	Adiol	Bdiol	T/E	Adiol/ Bdiol	And/ Etio	And/ T	And/ E
			[ng/ml]	[ng/ml]	[ng/ml]	[ng/ml]	[ng/ml]	[ng/ml]					
P04/10	14.11.06	1.024	3891	2126	10	84	146	195	**8.8**	0.75	1.83	46	393
P04/14	15.11.06	1.028	3248	2207	9	60	137	199	7.1	0.69	1.47	54	369
P04/27	29.11.06	1.025	3441	2546	7	52	139	242	6.4	0.58	1.30	66	461
P04/30	05.12.06	1.023	3304	1999	7	55	124	198	7.5	0.62	1.59	60	476
							mean		**7.0**	0.6	1.5	60.1	435.1
							sd		**0.6**	0.1	0.1	5.7	58.1
							cv [%]		**8.2**	9.2	10.0	9.5	13.4
							mean + 30%		**9.1**				

Tab. 1: Steroid profile parameters of volunteer P4 on different days during the T-gel application. The data can lead to the misinterpretation:" Naturally elevated T/E ratio based on a low E excretion".

To prevent such misinterpretation, i.e. false negative results, GC/C/IRMS analyses have to be performed.

3.3 GC/C/IRMS results

The delta value of testosterone in the T-gel is –27.0 ± 0.07 (n=5) (2).
At the moment only urine samples of 2 volunteers (P10 and P9) have been analysed with GC/C/IRMS. As target compounds were choosen And, Etio and T for volunteer P10 and additionally Adiol and Bdiol for volunteer P9. As internal reference compounds 11-hydroxy-androsterone (11OHA) and pregnanediol were analysed.

In Figure 18 T/E values and the corresponding GC/C/IRMS results (difference of delta values of the target compounds and the internal reference compounds) of urine samples of volunteer P10 before, during and after the T-gel application are presented. All 6 T/E values during the application exceeded the WADA cut off limit of 4. The GC/C/IRMS analysis only delivered 4 adverse findings, i.e. delta/delta differences above 3 (8), when And was chosen as target compound. The analysis of testosterone as target compound delivered adverse findings, i.e. delta/delta differences > 3, for all samples "on T-gel".

In Figures 19-21 T/E values and the corresponding GC/C/IRMS results of urine samples of volunteer P9 before, during and after the T-gel application are presented. Volunteer P9 has performed an intermittent application of T-gel. With the target compound And, the delta/delta differences did not exceed the value of 3, i.e. the administration of T-gel was not detected with GC/C/IRMS (Fig. 19). The analysis of T and Adiol as target compounds led to 2 and 3 adverse analytical findings respectively (Fig. 20). As shown in Figure 21 the delta/delta differences between Bdiol and 11OHAN are much smaller than the differences between Adiol and 11OHAN. These results once again demonstrate that T is converted mainly to 5-alpha-steroids after transdermal application, due to the high 5-alpha-reductase activity of the skin.
The results of the GC/C/IRMS analyses show that for the detection of the misuse of T-gel the target compounds T and Adiol are much more suitable than And, Etio and Bdiol.

Fig. 18: T/E ratios and GC/C/IRMS data (delta/delta-differences) of urine samples of vounteer P10 before, during and after a 6 week continuos application of testosterone gel (red bars: values during application, blue line in IRMS figures: delta/delta difference of 3)

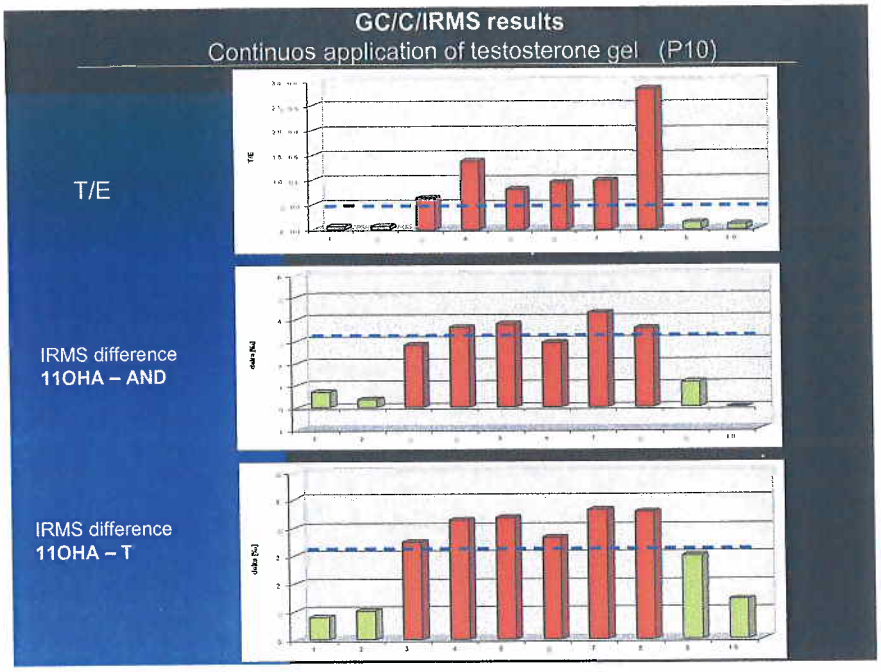


Fig. 19: T/E ratios and GC/C/IRMS data (delta/delta-differences) of urine samples of volunteer P9 before, during and after a 6 week intermittent application of testosterone gel (red bars: values during application, blue line in IRMS figures: delta/delta difference of 3)

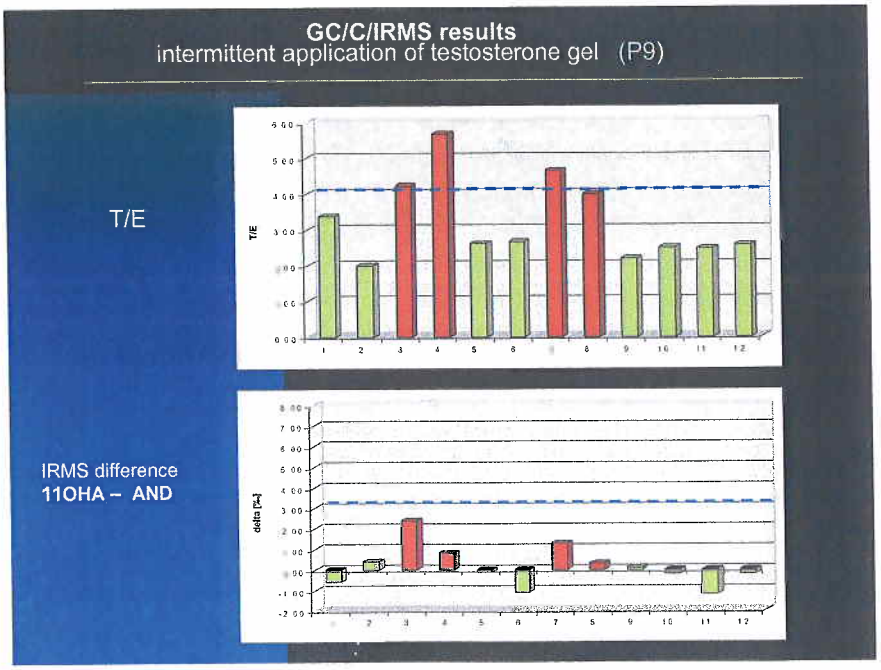


Fig. 20: GC/C/IRMS data (delta/delta-differences) of urine samples of volunteer P9 before, during and after a 6 week intermittent application of testosterone gel (red bars: values during application, blue line: delta/delta difference of 3)

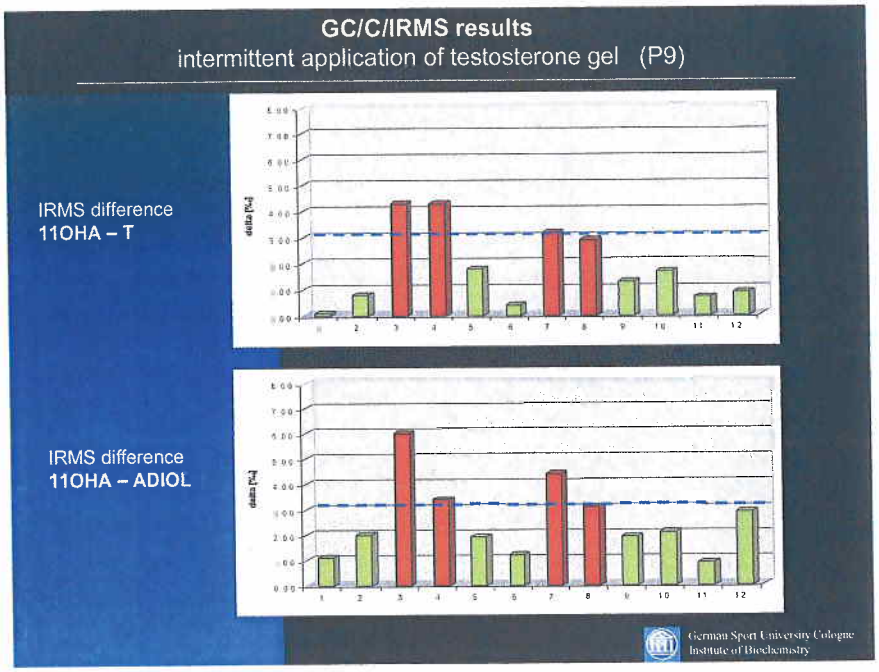
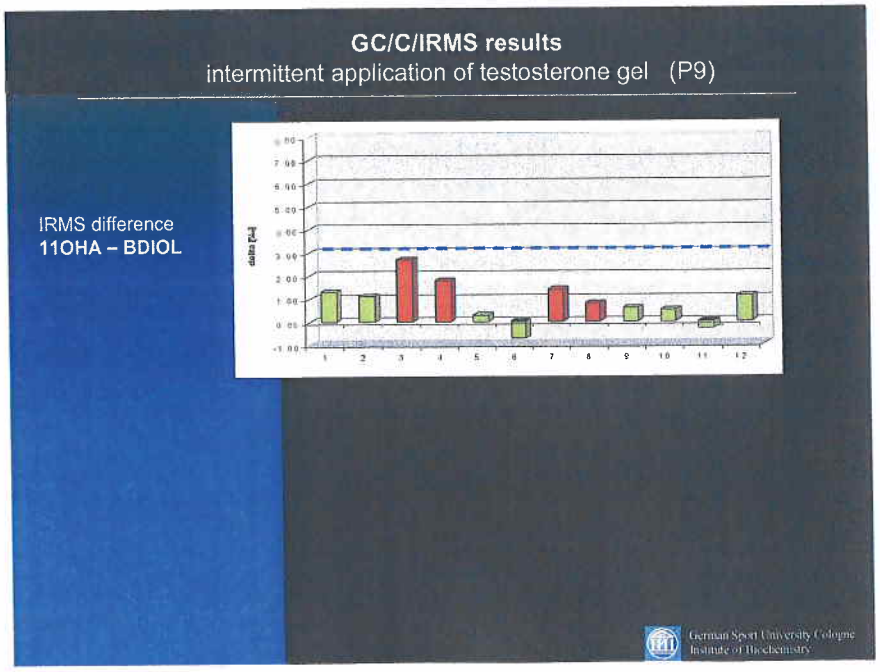


Fig. 21: GC/C/IRMS data (delta/delta-differences) of urine samples of volunteer P9 before, during and after a 6 week intermittent application of testosterone gel (red bars: values during application, blue line: delta/delta difference of 3)



4 Conclusions

According to the actual results of the investigation the following can be concluded:

- The misuse of testosterone gel can be detected by the use of individual reference ranges. Limits of population based reference ranges (e.g. T/E > 4) are not sufficient.
- The most discriminating parameters of the steroid profile are the ratios T/E, Adiol/E, And/E.
- For IRMS the most discriminating analytes are T and Adiol, whereas AND, ETIO and Bdiol are more influenced by endogenous production

5 Next steps

The next steps of this research project will be:

- Improvement of the sample preparation for the GC/C/IRMS analyses of Adiol
- Establishment of GC/C/IRMS reference values for the delta/delta differences of the target compounds T and Adiol and different internal reference steroids
- Statistical evaluation of the data

6 References

1. Swerdloff, R.S., Wang, C., Cunningham, G., Dobs, A., Iranmanesh, A., Matsumoto, A.M., Snyder, P., Weber, T., Longstreth, J., Berman, N.: Longterm pharmakokinetics of transdermal testosterone gel in hypogonadal men. J Clin Endocrinol Metab 85 (2000) 4500-10

2. Geyer, H., Flenker, U., Mareck, U., Sommer, F., Schänzer, W.: Preliminary results regarding the detection of the misuse of testosterone gel. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (12). Sport und Buch Strauß, Köln (2004) 121-127

3. Donike, M., Ueki, M., Kuroda, Y., Geyer, H., Nolteernsting, E., Rauth, S., Schänzer, W., Schindler, U., Völker, E., Fujisaki, M.: Detection of dihydro-testosterone (DHT) doping - Alterations in the steroid profile and reference ranges for DHT and its 5α-metabolites. J. Sports.Med.Phys.Fitness, 35 (1995) 235-50.

4. Geyer, H., Schänzer, W., Mareck-Engelke, U., Nolteernsting, E., Opfermann, G.: Screening procedure for anabolic steroids-The control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (5). Sport und Buch Strauß, Köln (1998) 99-102

5. Flenker, U., Horning, S., Nolteernsting, E., Geyer, H., Schänzer, W.: Measurement of $^{13}C/^{12}C$-Ratios to Confirm Misuse of Endogenous Steroids. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (6). Sport und Buch Strauß, Köln (1999) 243-256

6. Flenker, U., Nolteernsting, E., Geyer, H., Schänzer, W.: A Screening method for synthetic testosterone and its precursors based on GC/C/IRMS. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (7). Sport und Buch Strauß, Köln, (1999) 241-248

7. Schänzer, W., Geyer, H., Mareck, U.: Changes of steroid profile parameters after repeated oral application of testosterone undecanoate. Unpublished results (1996)

8. WADA technical document TD2004EAAS, 2004

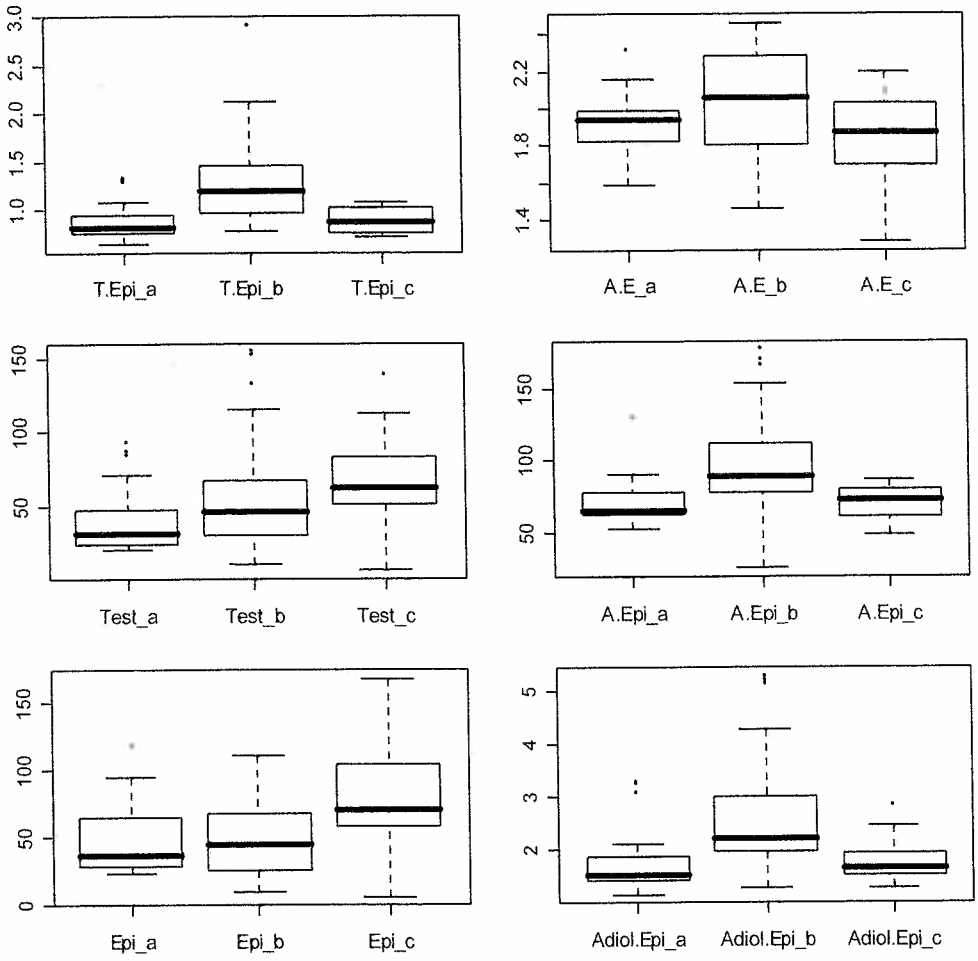
9. Geyer, H., Mareck-Engelke, U., Schänzer, W., Donike, M.: The Cologne protocol to follow up high testosterone/ epitestosterone ratios. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (4). Sport und Buch Strauß, Köln , (1997) 107-126.

7 Annex

Annex 1: Boxplots of selected steroid profile parameters and scatter diagrams of the T/E ratios of all 18 volunteers

Annex 1

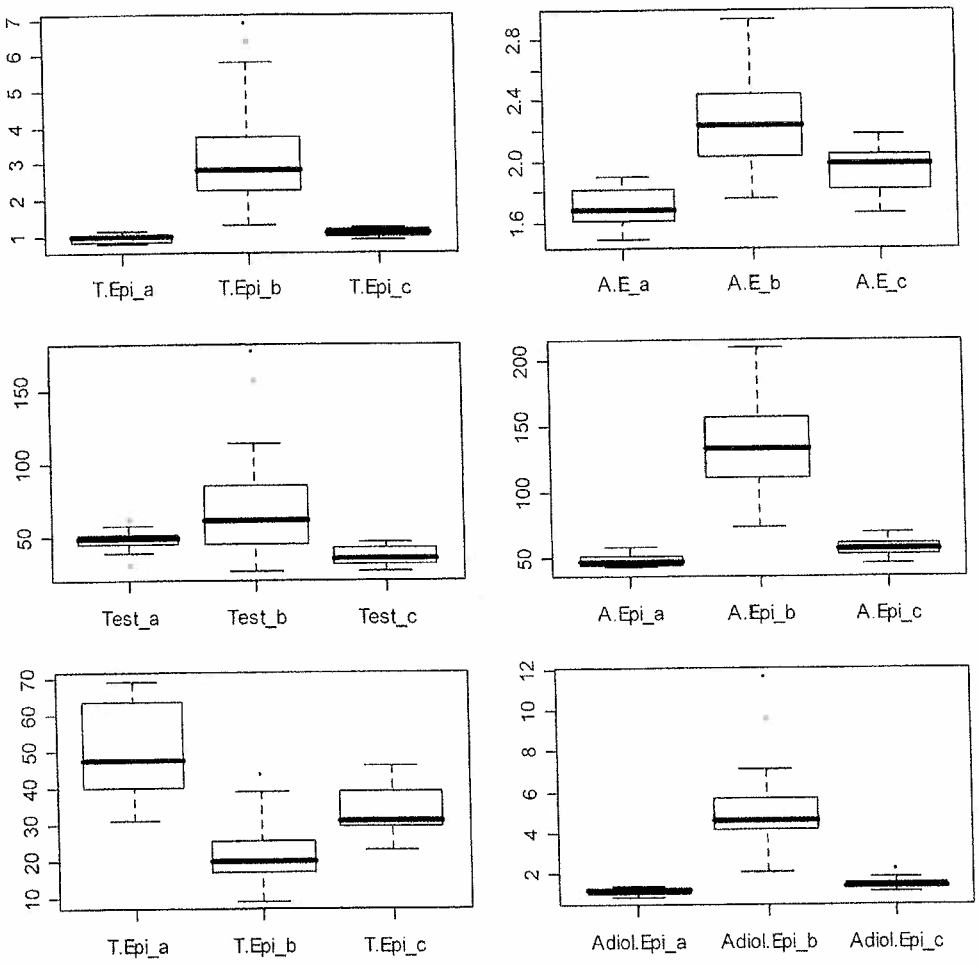
Person 1
Testosteronegel Application



_a	before Testogel application	n = 25
_b	during Testogel application	n = 45
_c	post Testogel application	n = 15

T.Epi	Testosterone/Epitestosterone ratio
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone ratio
A.Epi	Androsterone/Epitestosterone ratio
Adiol.Epi	5a-Androstanediol/Epitestosterone ratio

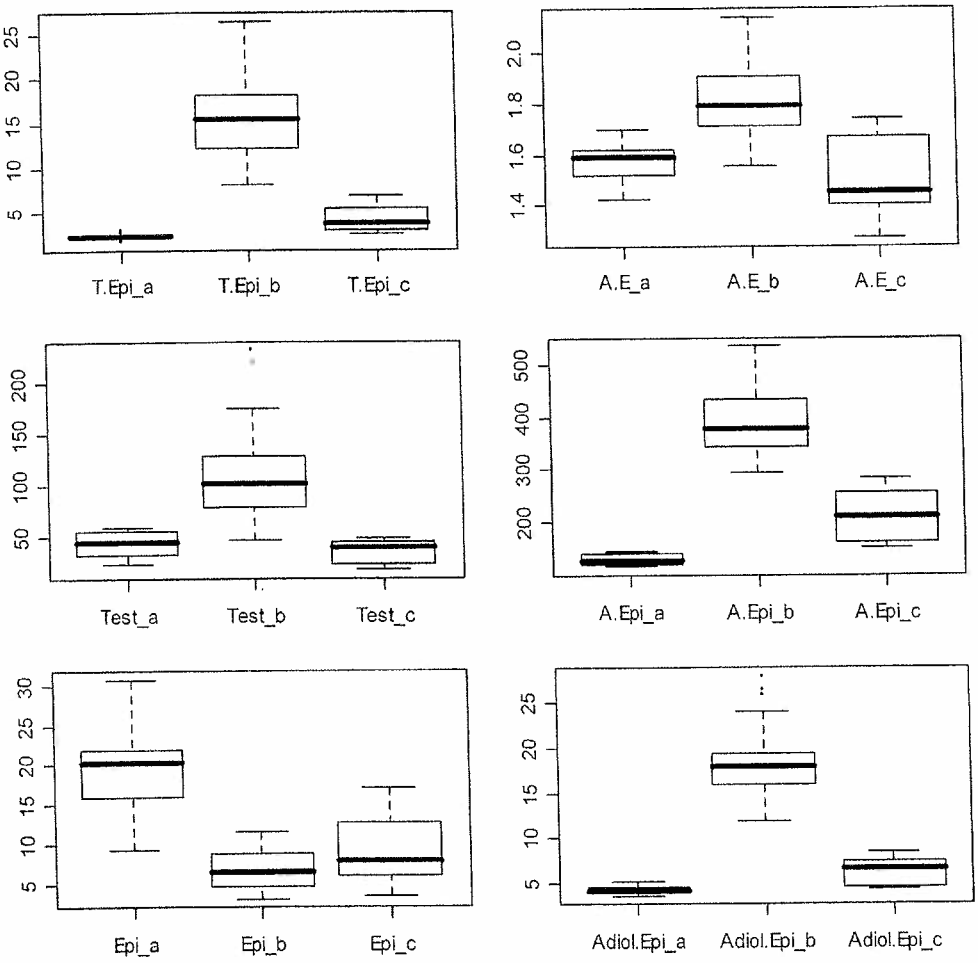
Person 2
Testosteronegel Application



_a	before Testogel application	n = 10
_b	during Testogel application	n = 33
_c	post Testogel application	n = 11

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

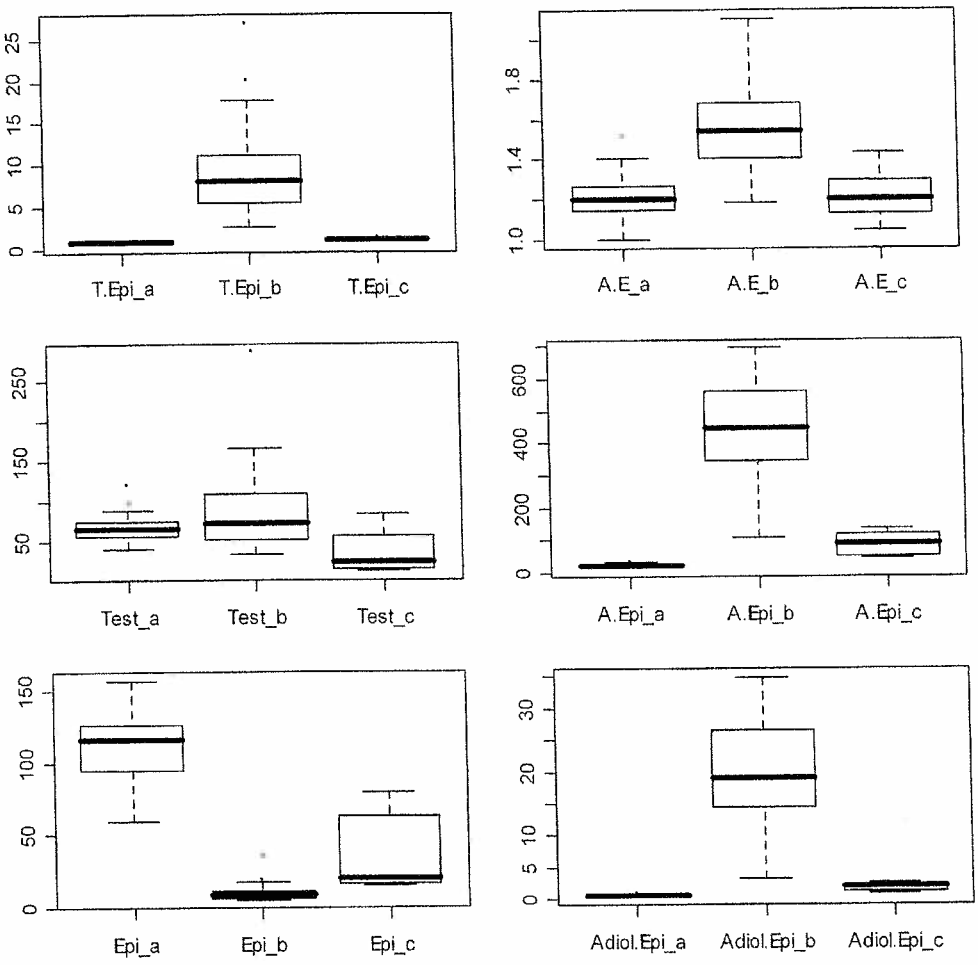
Person 3
Testosteronegel Application



_a	before Testogel application	n = 13
_b	during Testogel application	n = 38
_c	post Testogel application	n = 10

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

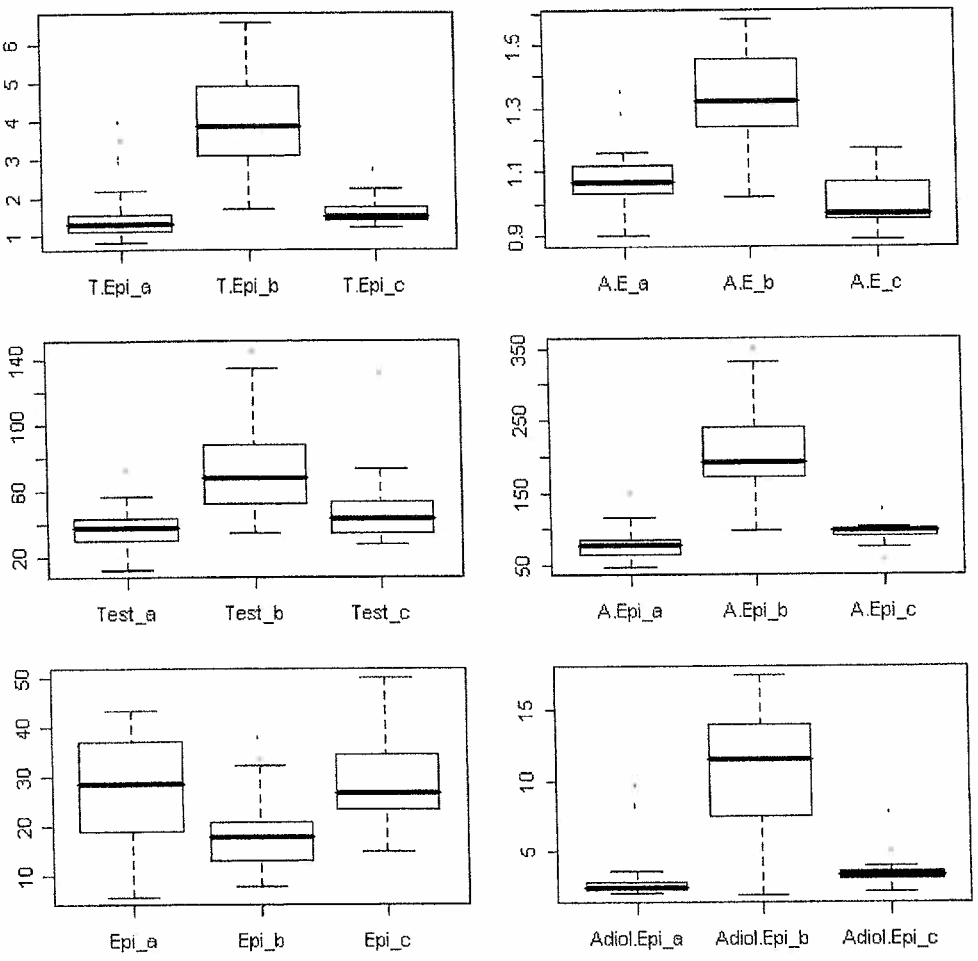
Person 4
Testosteronegel Application



_a	before Testogel application	n = 25
_b	during Testogel application	n = 42
_c	post Testogel application	n = 15

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

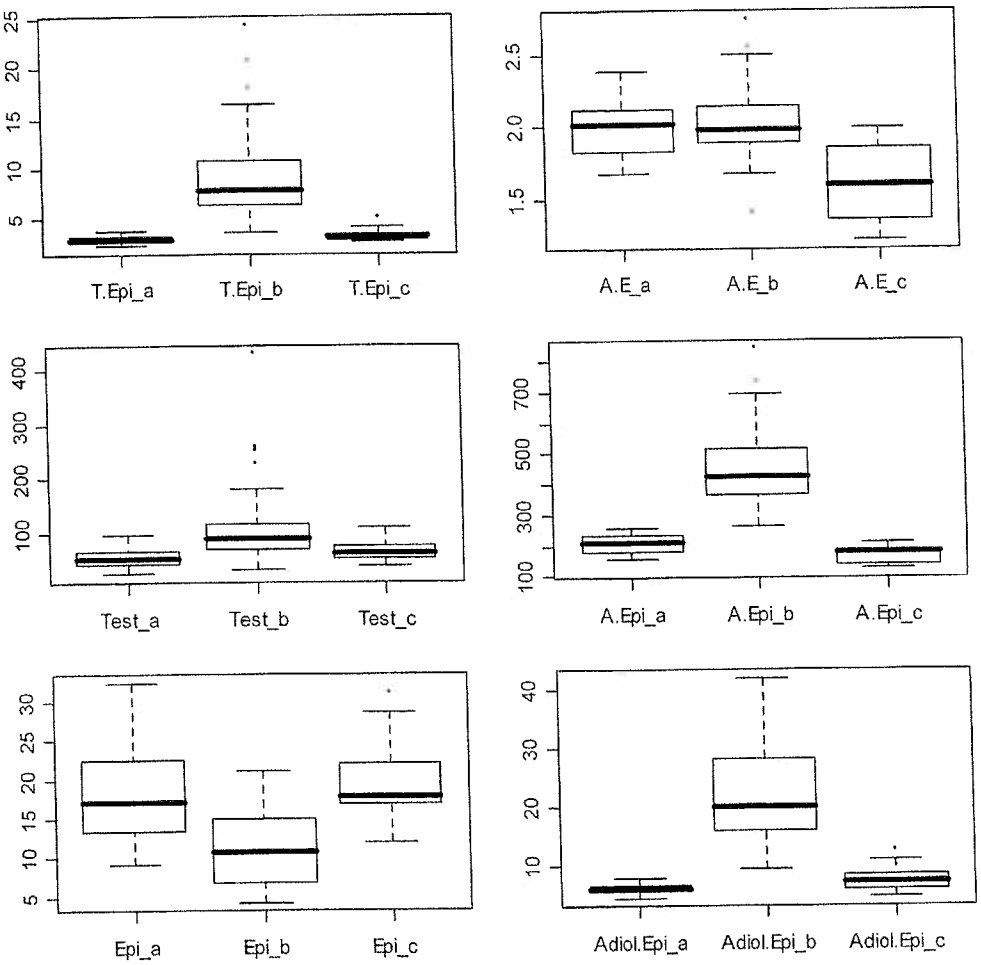
Person 5
Testosteronegel Application



_a	before Testogel application	n = 30
_b	during Testogel application	n = 39
_c	post Testogel application	n = 13

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

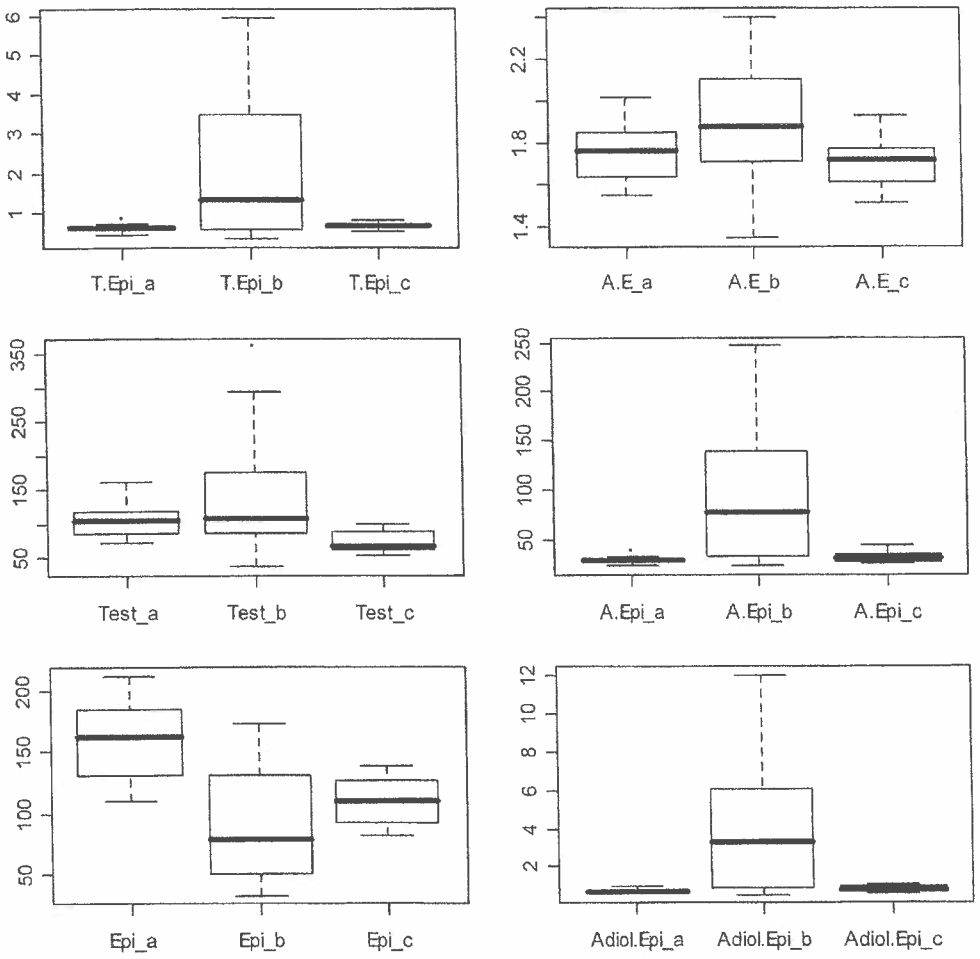
Person 6
Testosteronegel Application



_a	before Testogel application	n = 29
_b	during Testogel application	n = 46
_c	post Testogel application	n = 13

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

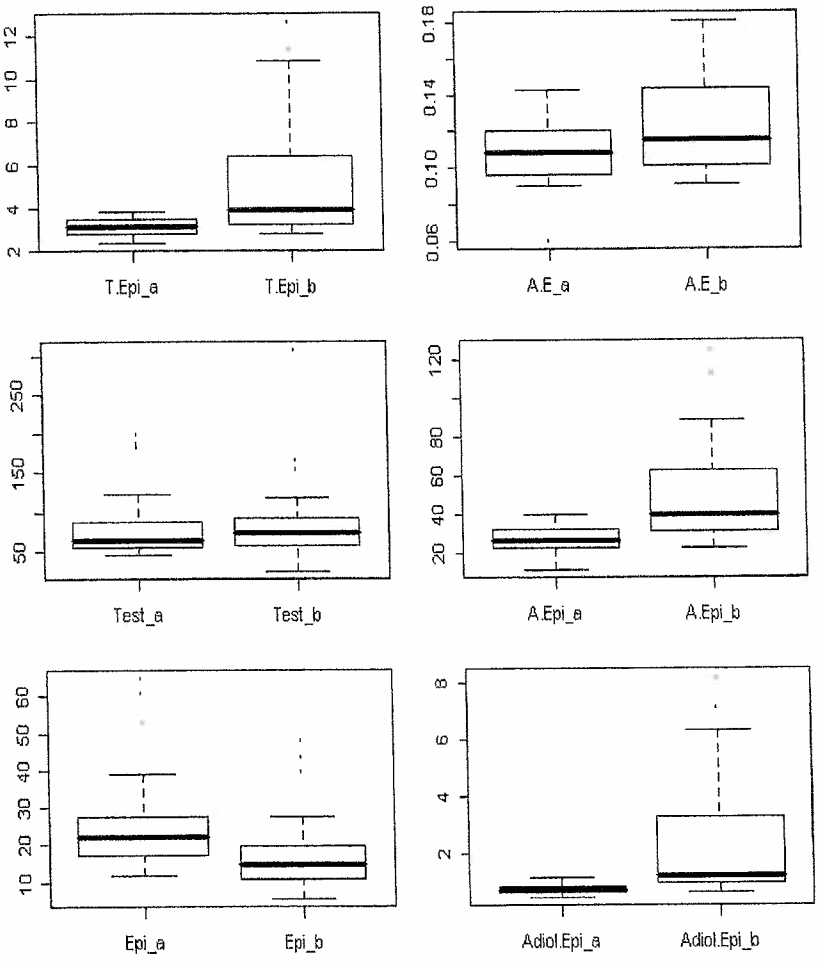
Person 7
Testosteronegel Application



_a	before Testogel application	n = 22
_b	during Testogel application	n = 33
_c	post Testogel application	n = 11

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

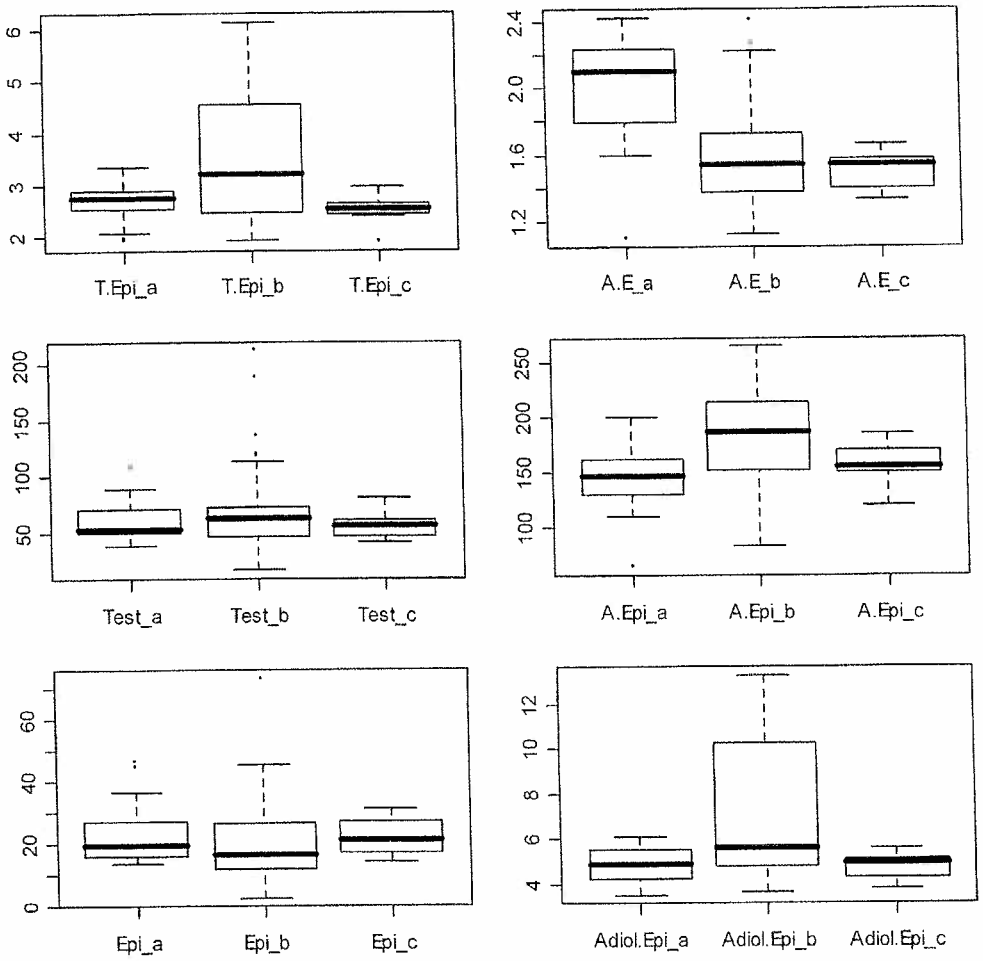
Person 8
Testosteronegel Application



_a	before Testogel application	n = 24
_b	during Testogel application	n = 30
_c	post Testogel application	n = 0

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

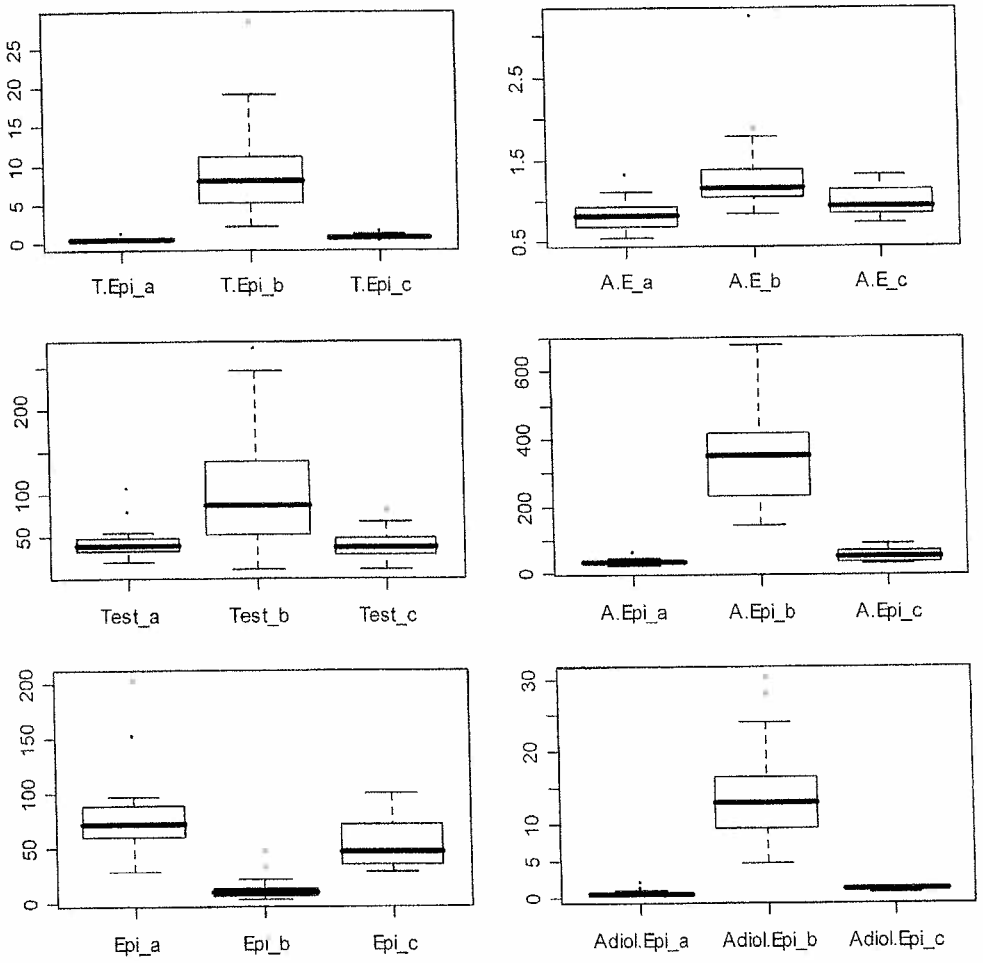
Person 9
Testosteronegel Application



_a	before Testogel application	n = 23
_b	during Testogel application	n = 37
_c	post Testogel application	n = 11

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

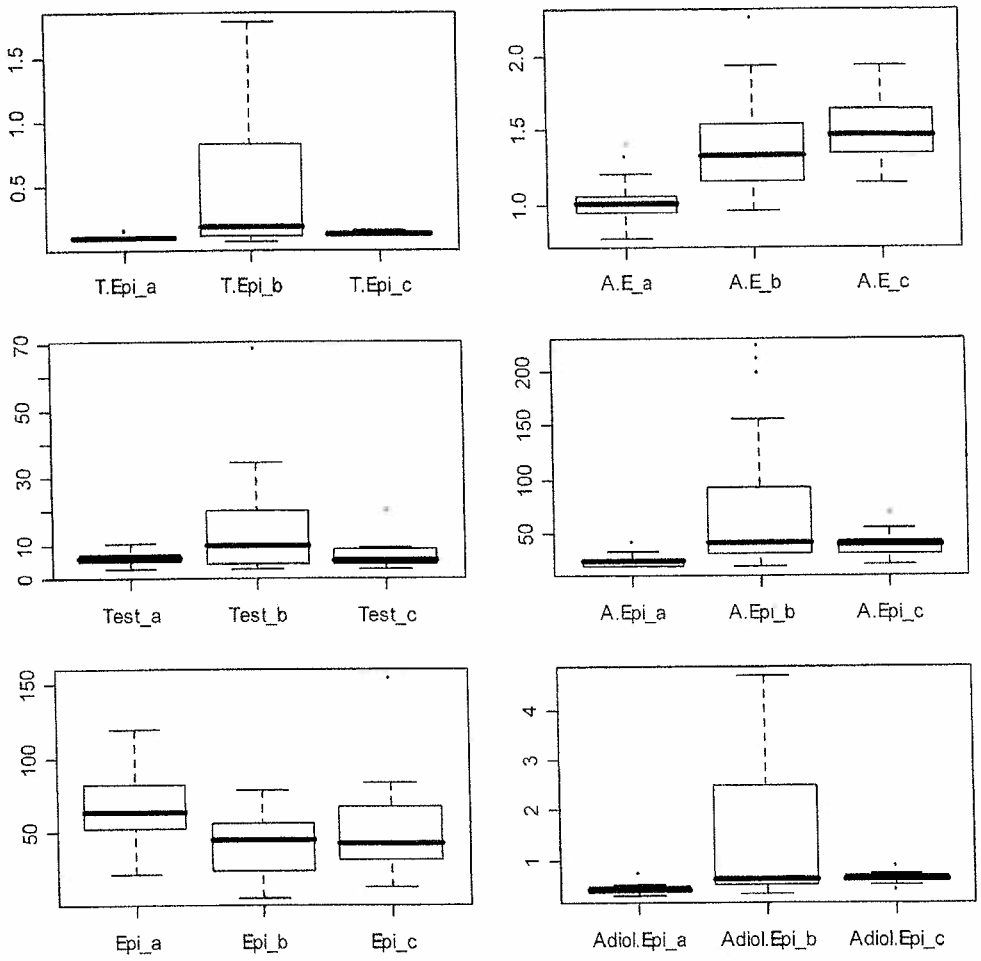
Person 10
Testosteronegel Application



_a	before Testogel application	n = 18
_b	during Testogel application	n = 33
_c	post Testogel application	n = 14

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

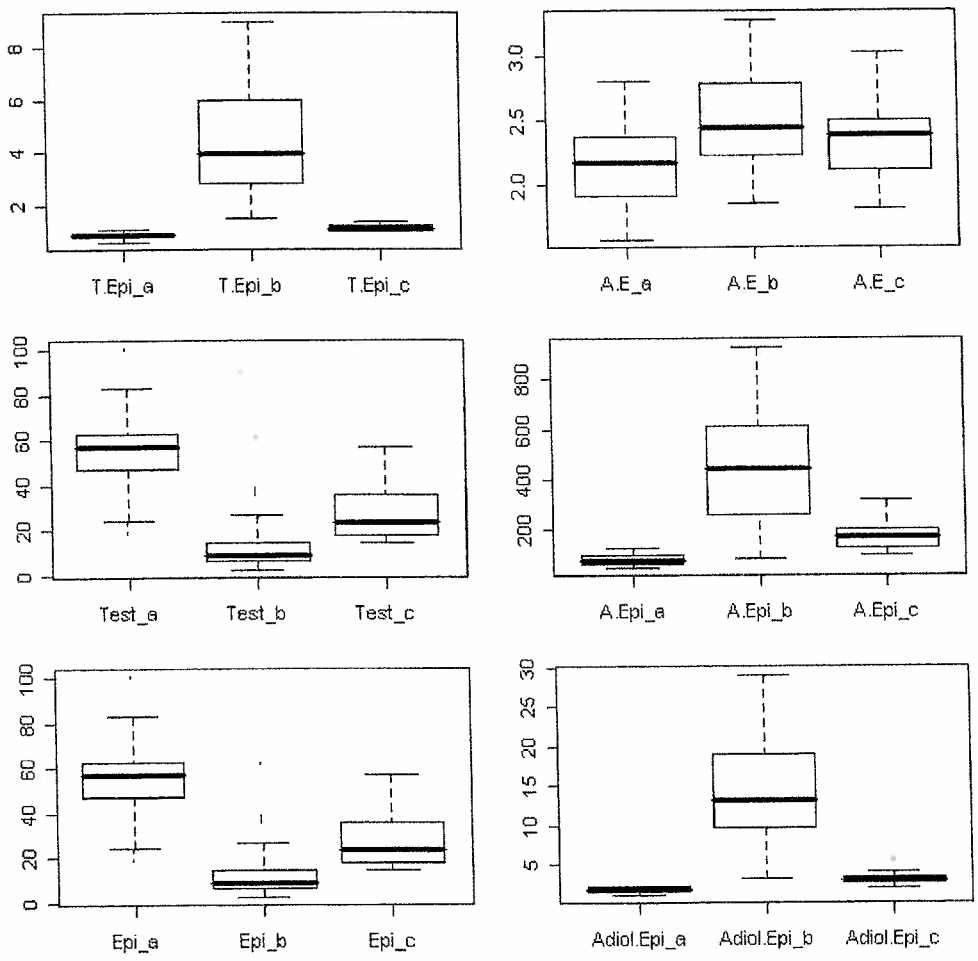
Person 11
Testosteronegel Application



_a	before Testogel application	n = 18
_b	during Testogel application	n = 44
_c	post Testogel application	n = 13

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

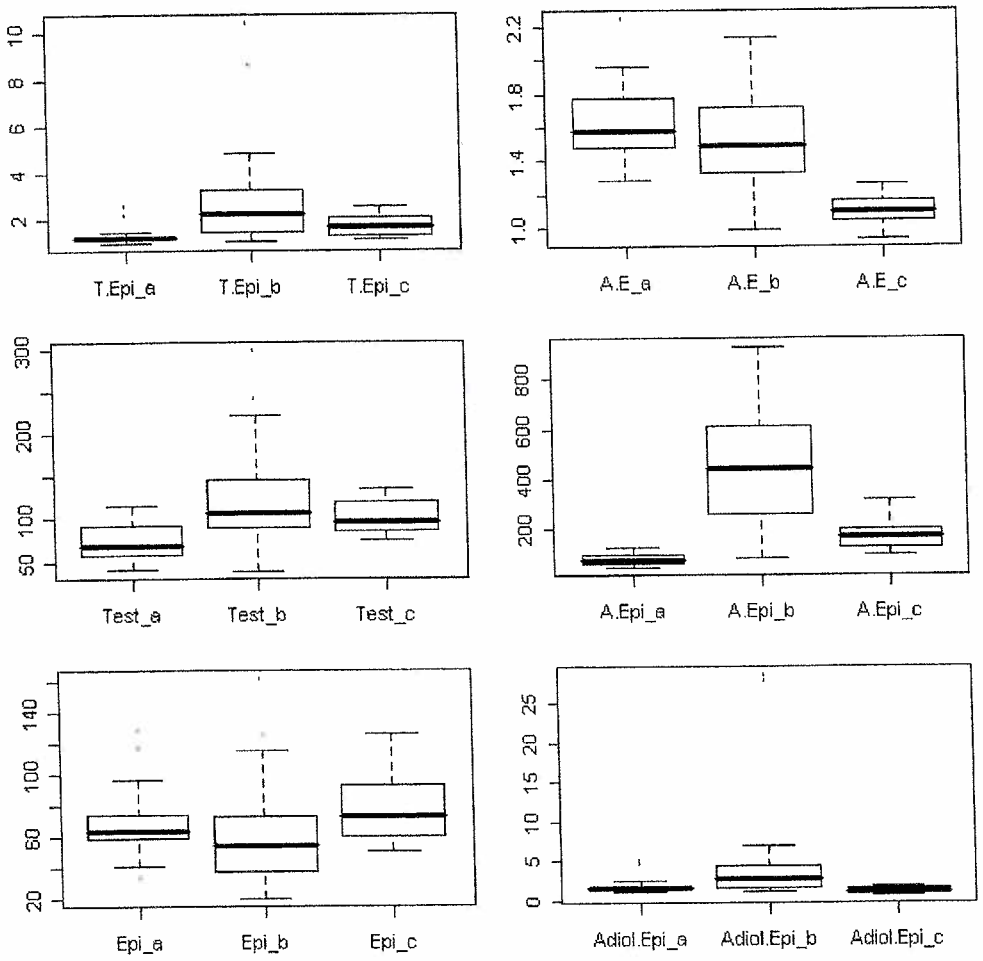
Person 12
Testosteronegel Application



_a	before Testogel application	n = 25
_b	during Testogel application	n = 40
_c	post Testogel application	n = 10

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

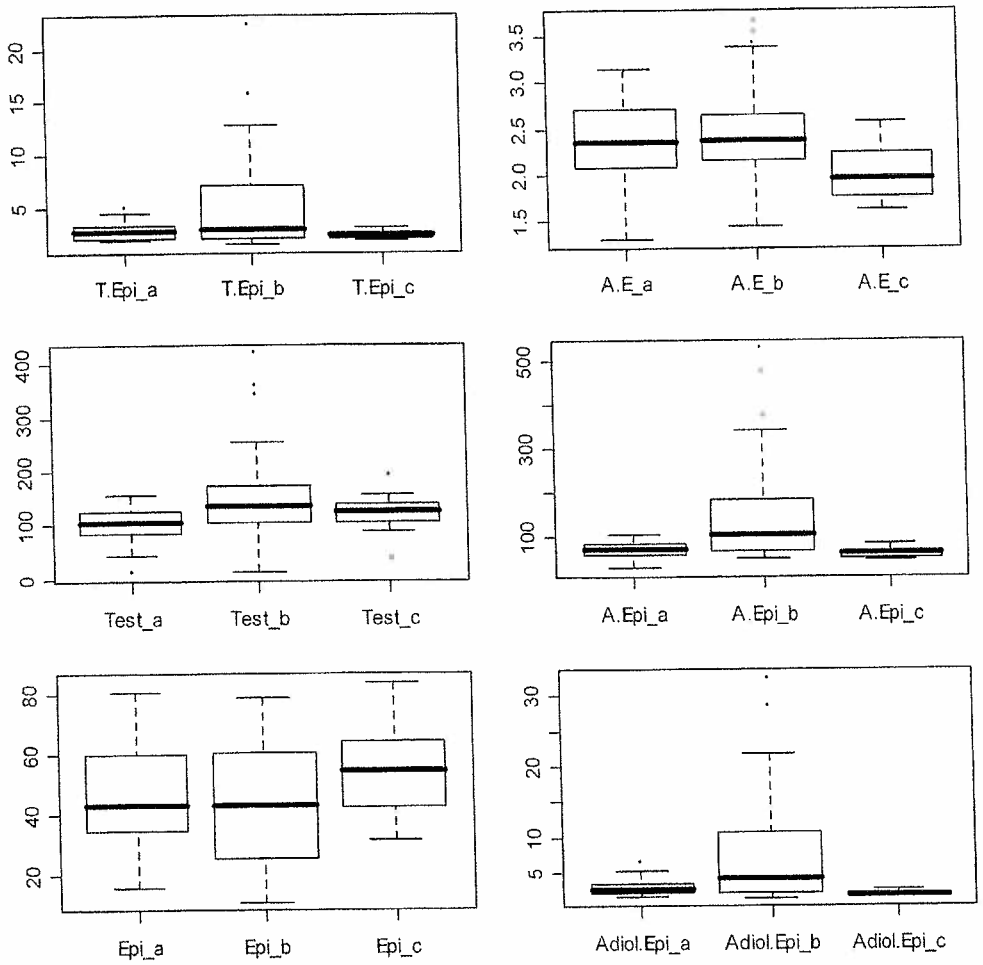
Person 13
Testosteronegel Application



_a	before Testogel application	n = 22
_b	during Testogel application	n = 37
_c	post Testogel application	n = 12

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

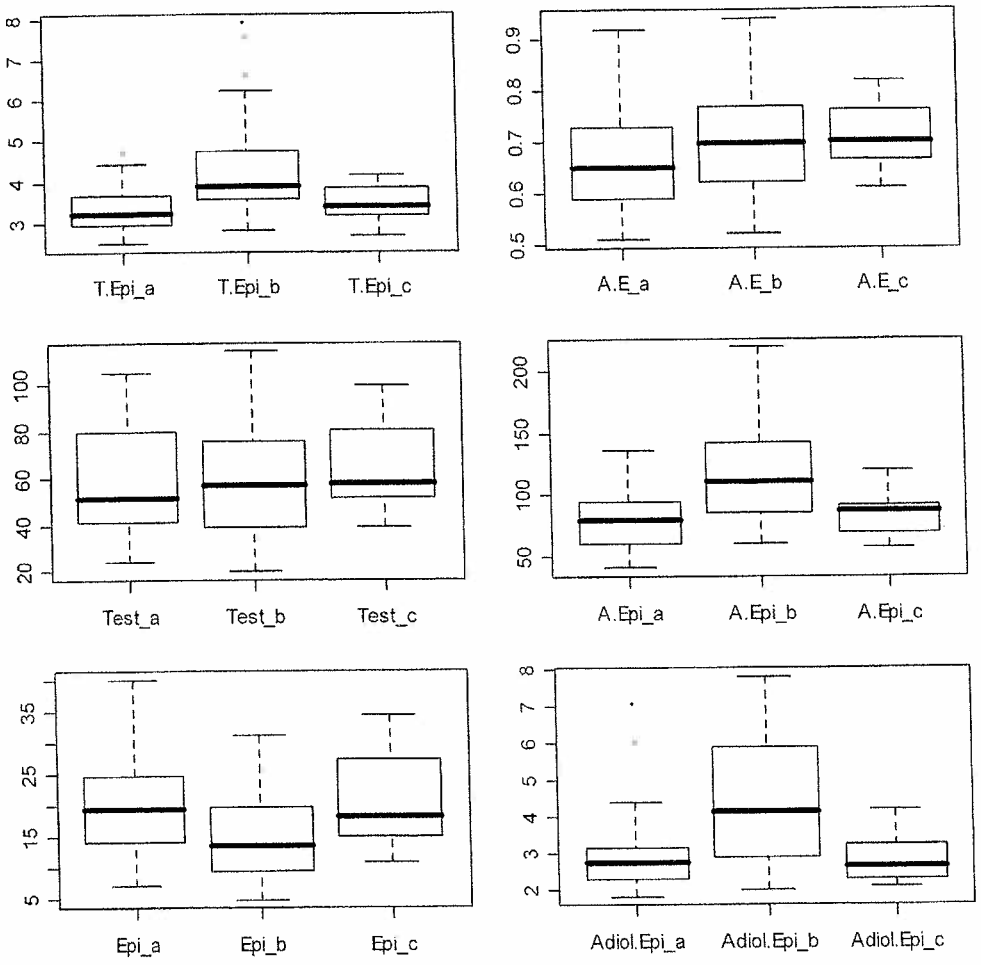
Person 14
Testosteronegel Application



_a	before Testogel application	n = 24
_b	during Testogel application	n = 44
_c	post Testogel application	n = 14

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

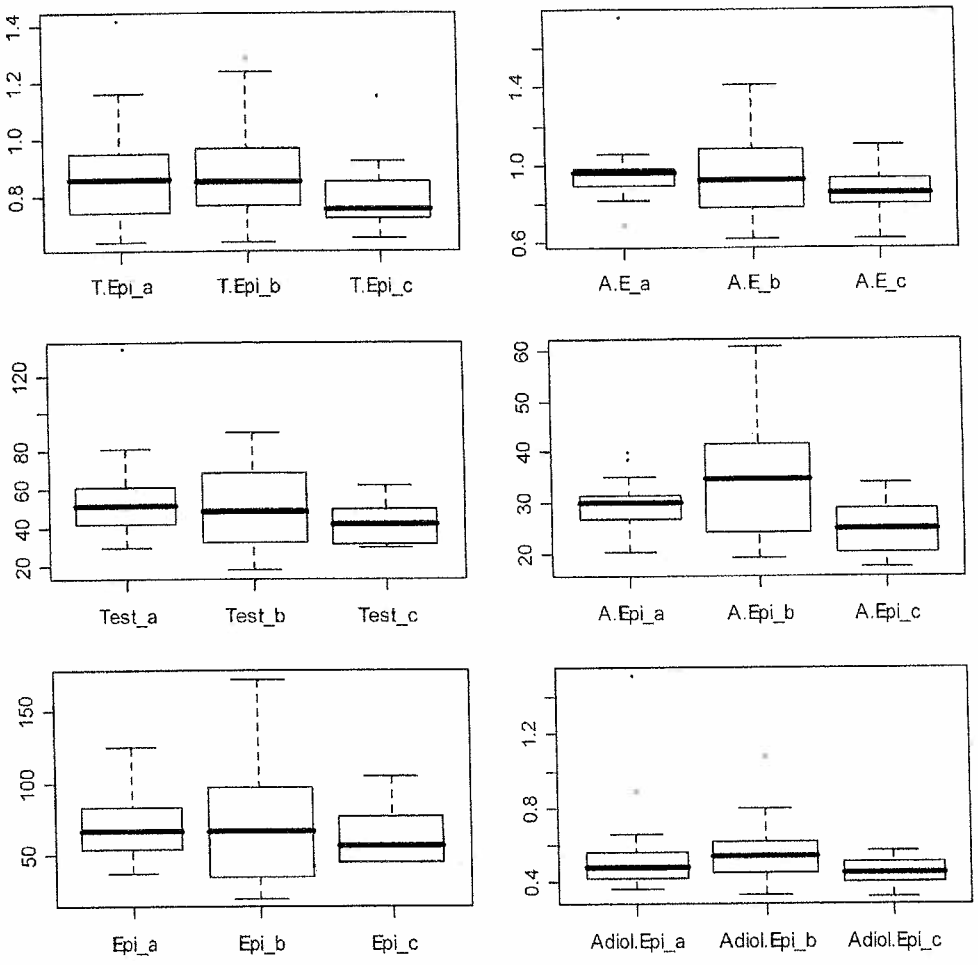
Person 15
Testosteronegel Application



_a	before Testogel application	n = 27
_b	during Testogel application	n = 44
_c	post Testogel application	n = 15

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

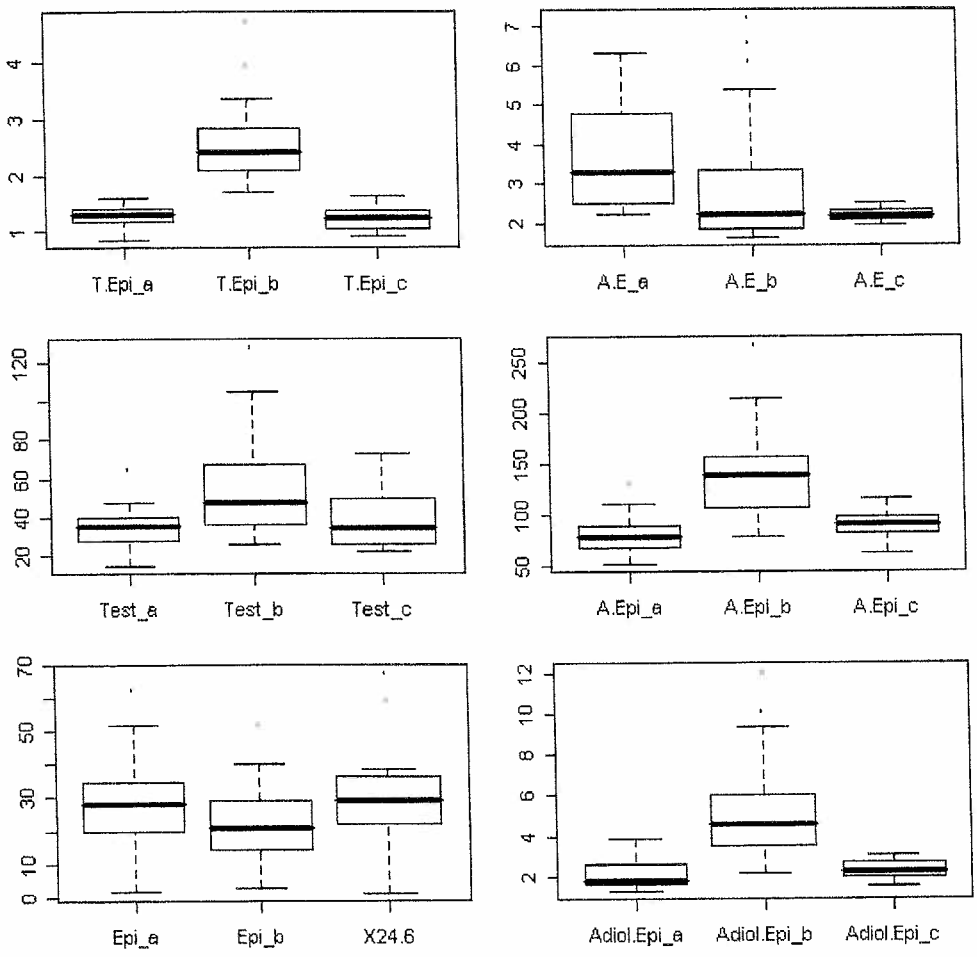
Person 16
Testosteronegel Application



_a	before Testogel application	n = 17
_b	during Testogel application	n = 29
_c	post Testogel application	n = 8

T.Epi	Testosterone/Epitestosterone quotient
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone quotient
A.Epi	Androsterone/Epitestosterone quotient
Adiol/Epi	5α-Androstanediol/Epitestosterone quotient

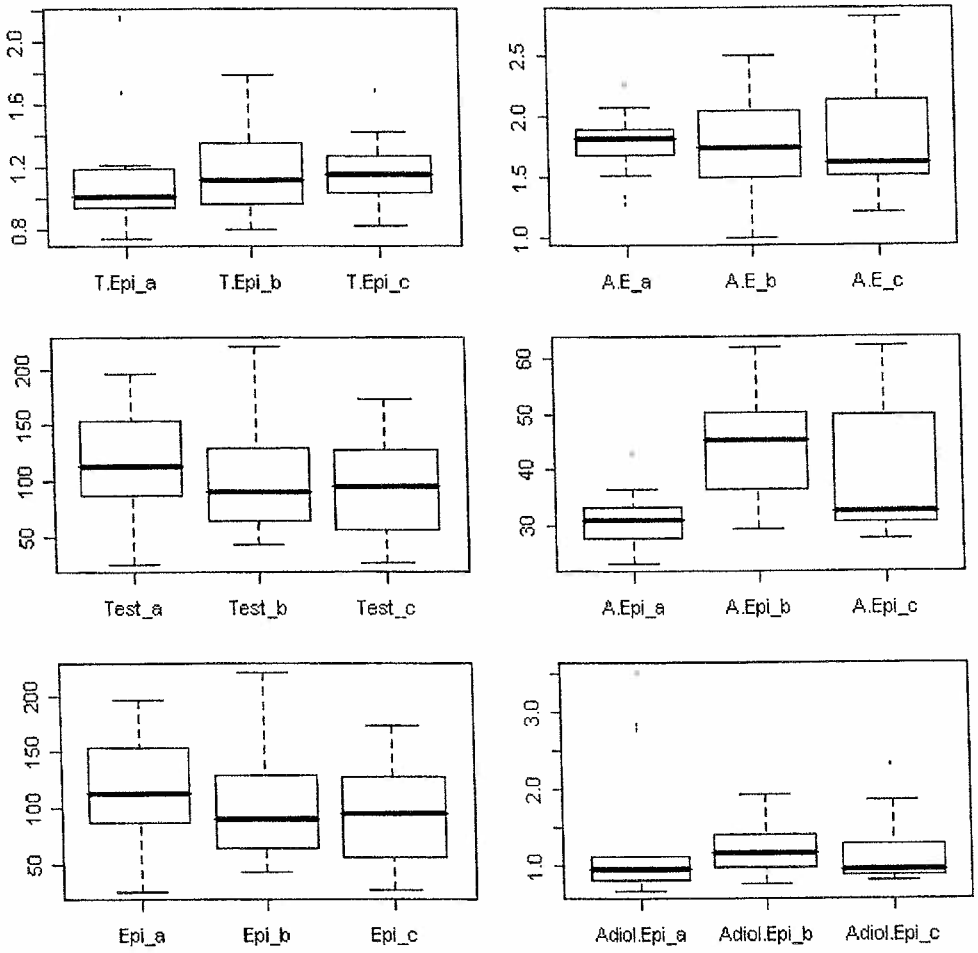
Person 17
Testosteronegel Application



_a	before Testogel application	n = 25
_b	during Testogel application	n = 30
_c	post Testogel application	n = 14

T.Epi	Testosterone/Epitestosterone ratio
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone ratio
A.Epi	Androsterone/Epitestosterone ratio
Adiol.Epi	5α-Androstanediol/Epitestosterone ratio

Person 18
Testosteronegel Application

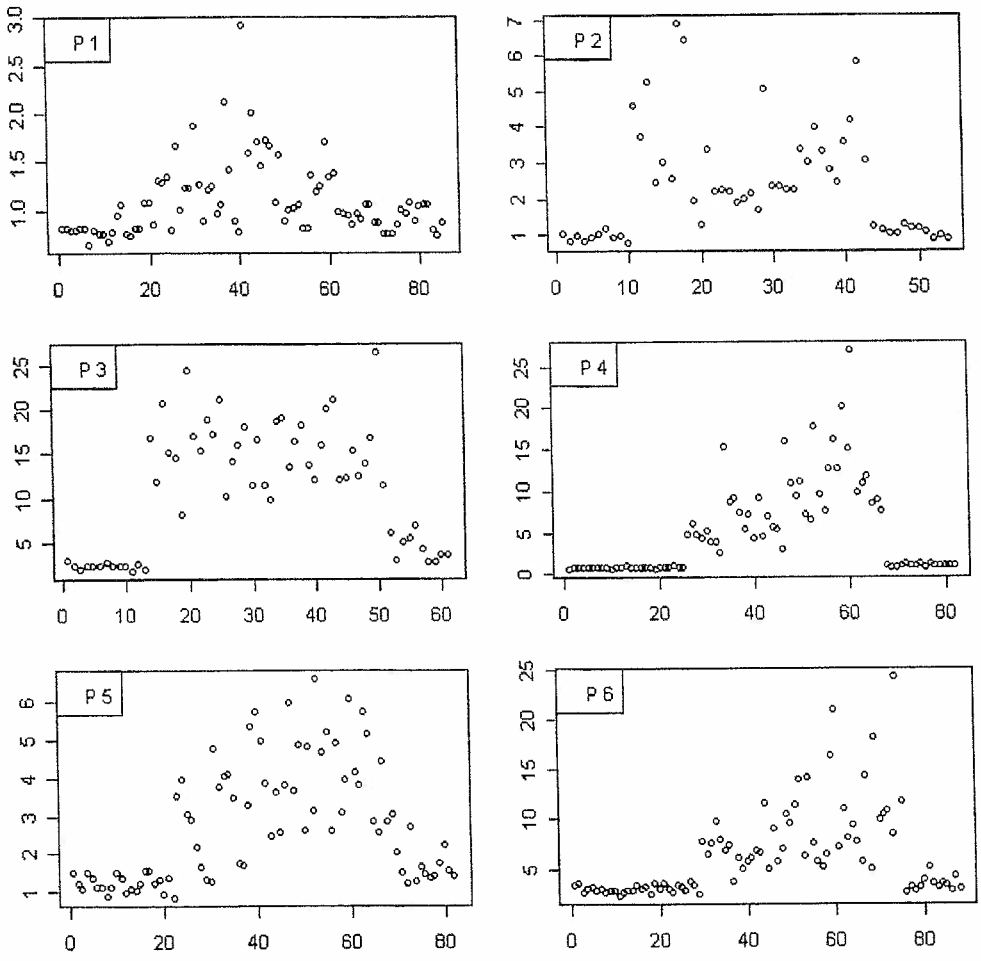


_a	before Testogel application	n = 18
_b	during Testogel application	n = 33
_c	post Testogel application	n = 12

T.Epi	Testosterone/Epitestosterone ratio
Test	Testosterone concentration (ng/ml)
Epi	Epitestosterone concentration (ng/ml)
A.E	Androsterone/Etiocholanolone ratio
A.Epi	Androsterone/Epitestosterone ratio
Adiol.Epi	5α-Androstanediol/Epitestosterone ratio

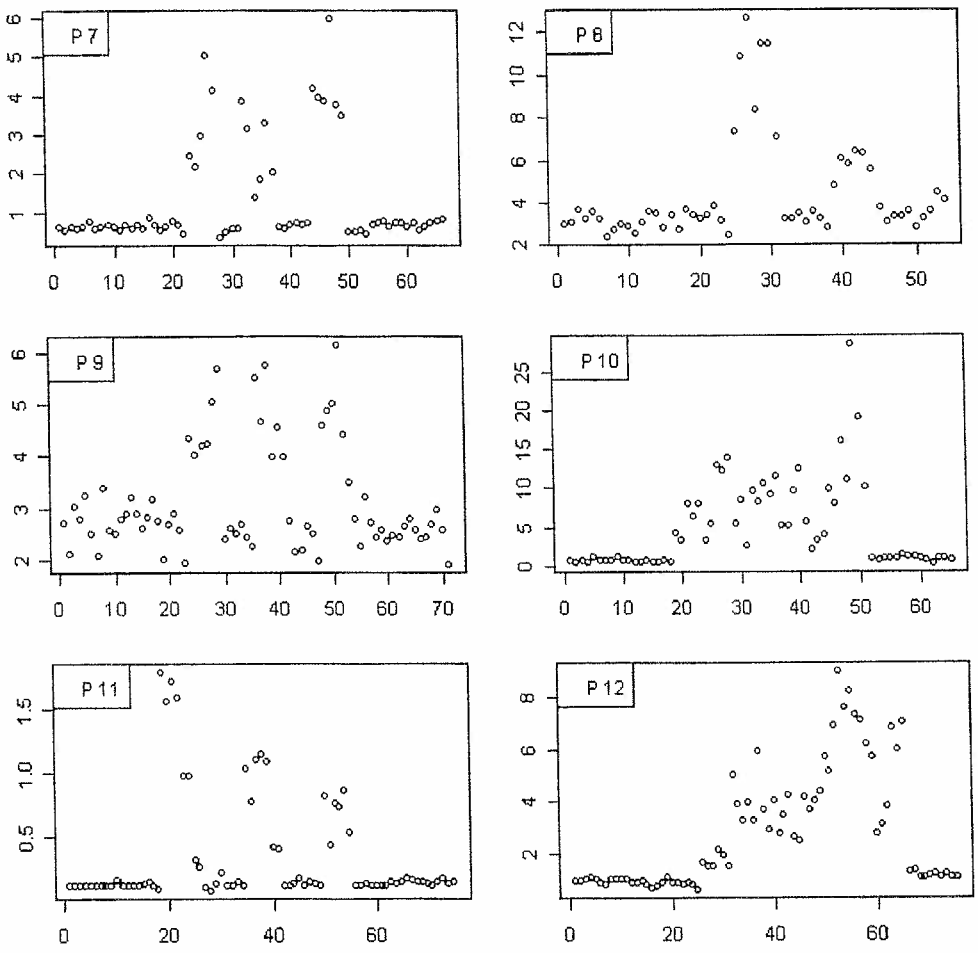
Person 1-6
Testosteronegel Application

T/E-Ratios



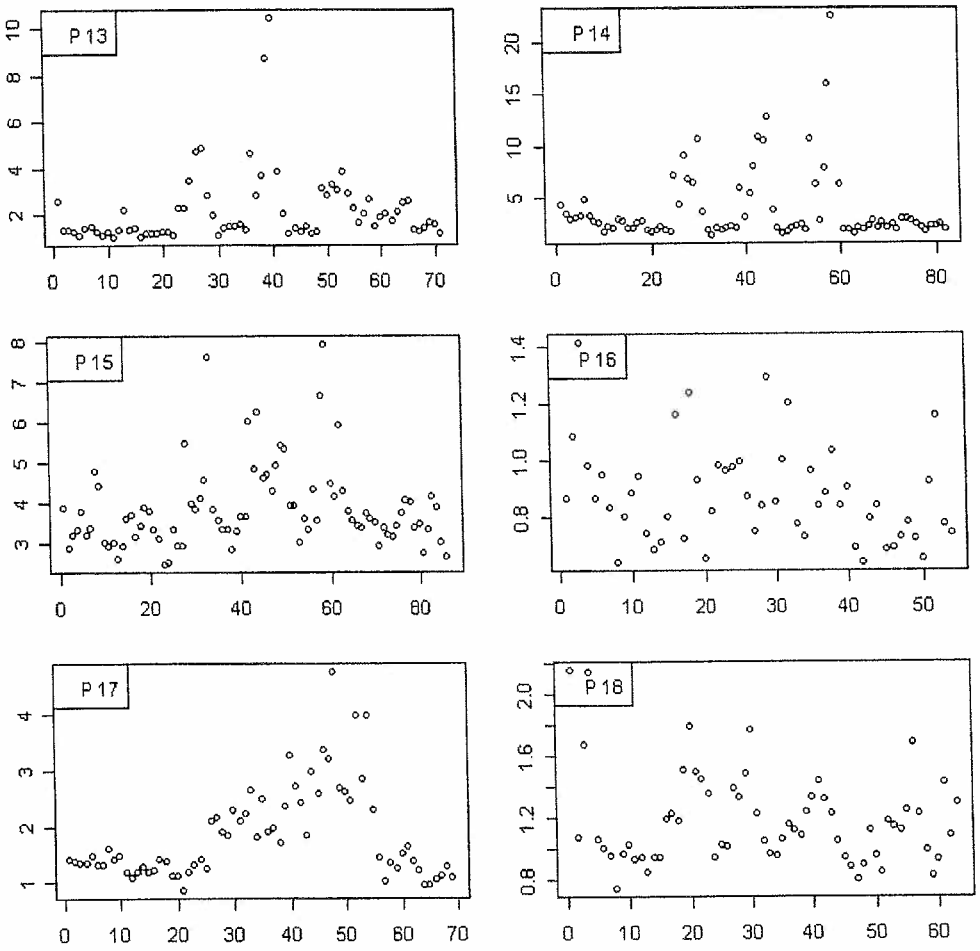
Person 7-12
Testosteronegel Application

T/E-Ratios



Person 13-18
Testosteronegel Application

T/E-Ratios

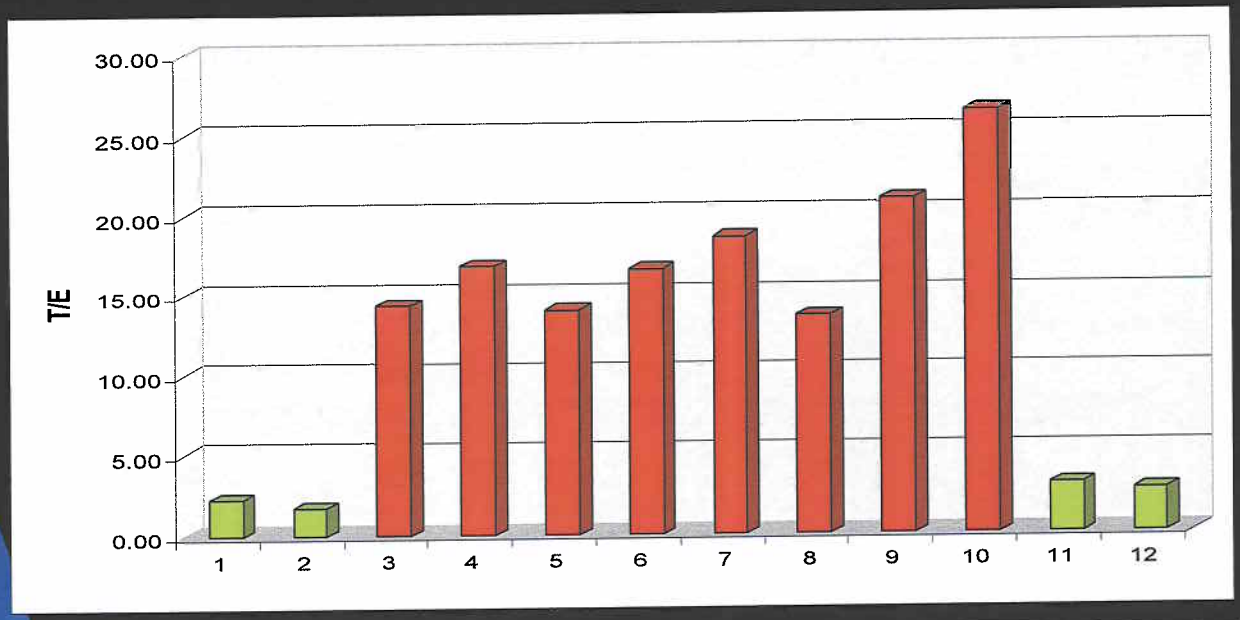


TAB 34A

GC/C/IRMS results
continuos application of testosterone gel (P3)

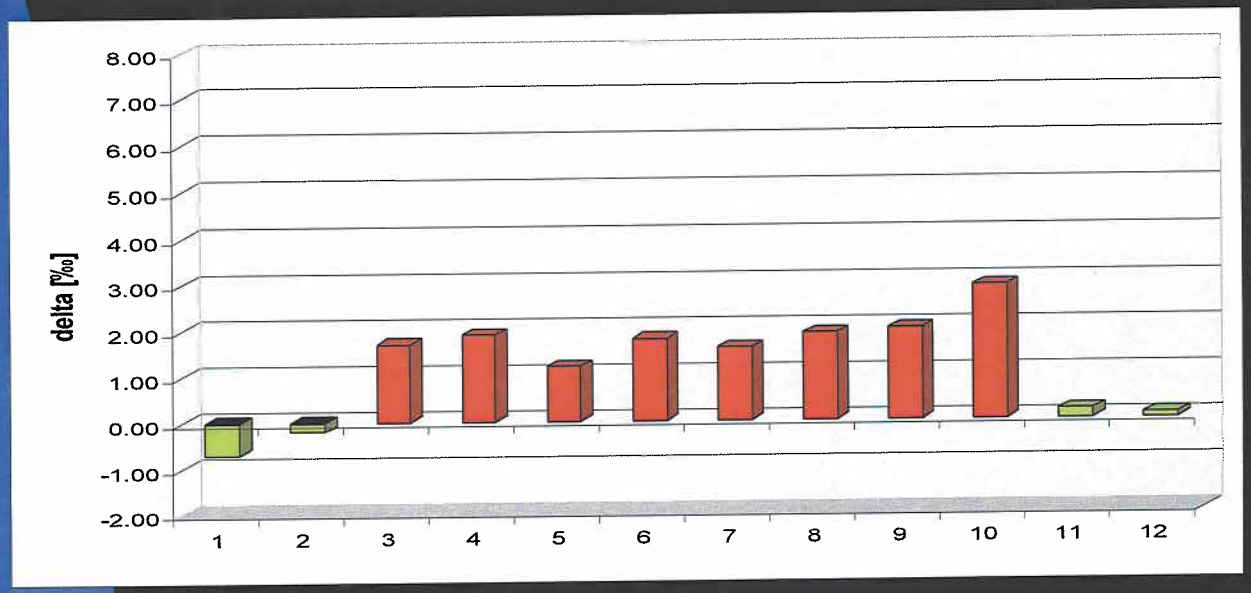
T/E

1



IRMS difference
PD - AND

2

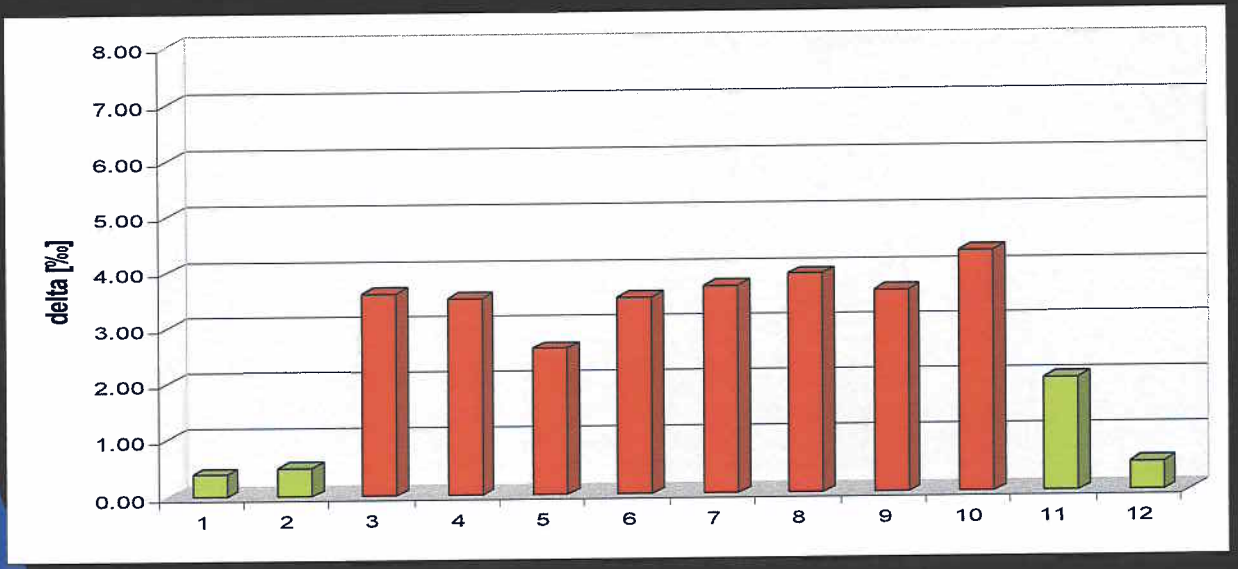


GC/C/IRMS results

continuos application of testosterone gel (P3)

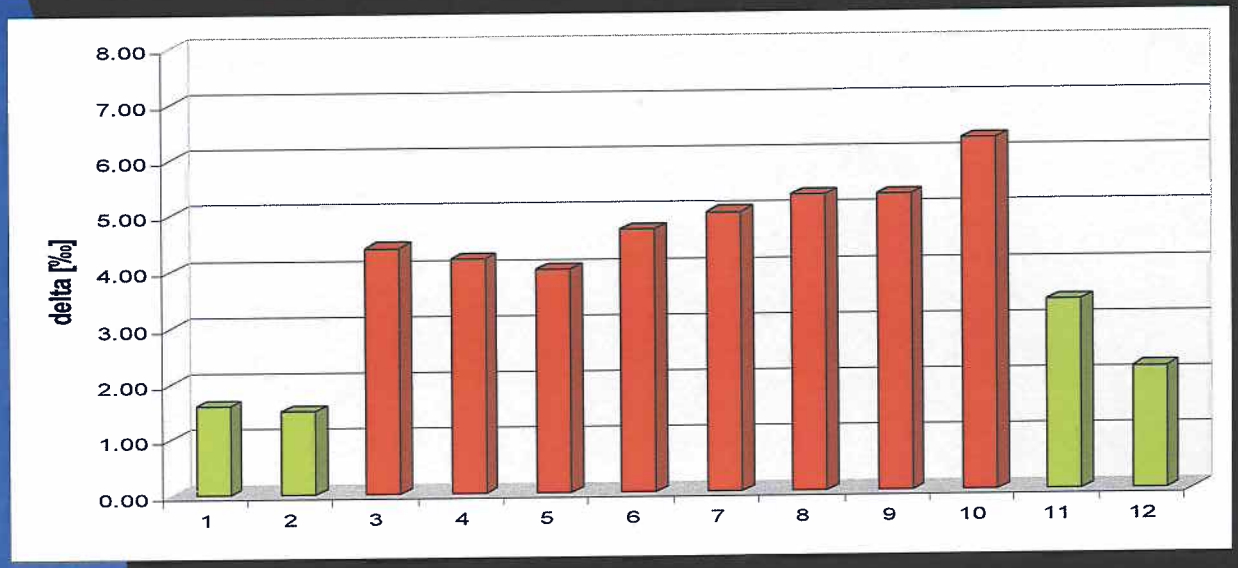
3

IRMS difference
PD - T



4

IRMS difference
PD - ADIOL

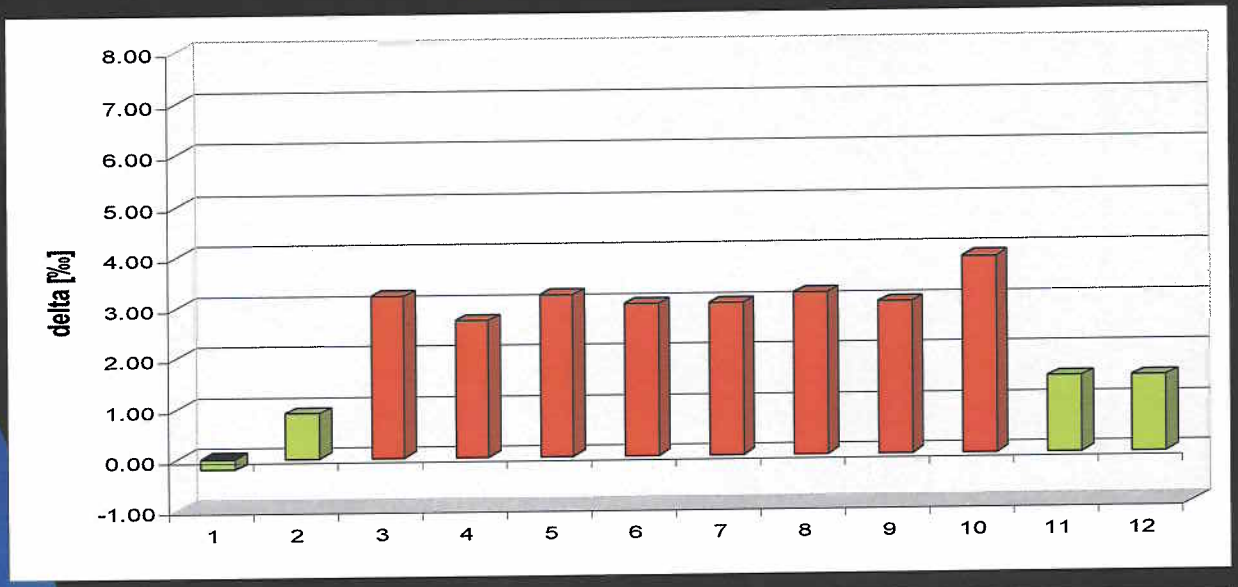


German Sport University Cologne
Institute of Biochemistry

GC/C/IRMS results
continuos application of testosterone gel (P3)

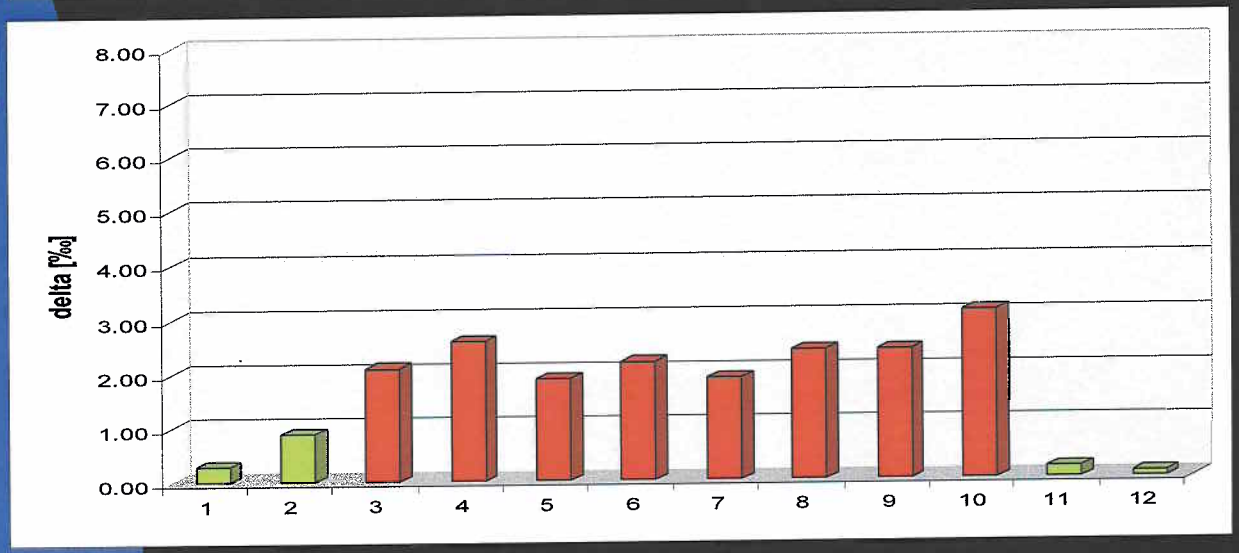
5

IRMS difference
PD - BDIOL



6

IRMS difference
PD - ETIO



German Sport University Cologne
Institute of Biochemistry

Explantion of data (volunteer Person 3)

Column 1,2 before testogel application

Column 3,10 during testogel application

Column 11,12 after testogel application

1. T/E ratio GC-MS data
2. IRMS delta differences **Preganediol** minus **Androsterone**
3. IRMS delta differences **Preganediol** minus **Testosterone**
4. IRMS delta differences **Preganediol** minus **5alpha-Androstanediol**
5. IRMS delta differences **Preganediol** minus **5beta-Androstanediol**
6. IRMS delta differences **Preganediol** minus **Etiocholanolone**

TAB 35

Final Report on WADA Grant

Pharmacokinetics of pharmaceutical Testosterone and TE in Subjects with Low and High Baseline TEs: The assessment of the ethnic differences and of the sensitivity of various carbon isotope ratio methods relative to TE.

Don H. Catlin, M.D.
UCLA Olympic Analytical Laboratory
September 30, 2006

1. EXECUTIVE SUMMARY

Our main findings are that HM (high baseline TE) and LM (low baseline TE) subjects differ both in the way they incorporate T into steroid metabolites and the way in which this incorporation is detected by TE and IRMS methodology. Using the TE method, T abuse is less likely to be detected in LM subjects than HM subjects. The TE method, even with a threshold of 4, would have missed 4 and possibly 5 of the 6 LM users.

The IRMS method, in particular the differences between both the 5α- and 5β-diol and the 5β-pdiol would have detected all subjects as T users. IRMS methodology is a superior detection method for T abuse because its effectiveness doesn't depend on baseline TE value and its retrospectivity is longer.

Contrary to expectations, E excretion rates increased during infusion. The $\delta^{13}C$ values of the excreted E approach that of the T used for the infusion suggesting that it arises from the infused material.

This study shows that male subjects differ remarkably in their ability to excrete T in urine and that this difference is positively correlated with their baseline TE ratio. The HM subjects respond to increasing doses of T by excreting more T in urine, and their excretion rates rise to 50μg/h. In contrast the LM subjects' capacity to excrete T is limited to approximately 3-4μg/h.

2A. BACKGROUND on CARBON ISOTOPE RATIO METHODS.

Over 98 percent of the carbon atoms in nature are ^{12}C but about 1.1% are ^{13}C, an isotope of carbon. If molecules of testosterone (T) or any other steroid are completely burned in a furnace at 800°C, the molecules are combusted to carbon dioxide and water. The gas chromatography-combustion-isotope ratio mass spectrometer (GC-C-IRMS) instrument measures the $^{13}C/^{12}C$ ratio of the combusted steroid, relative to a standard. The steroids are first extracted from urine, then separated in the GC, then combusted in the furnace, and finally the $\delta^{13}C$ value is determined.

There is a measurable difference in δ^{13} values between pharmaceutical T (e.g.-30‰) manufactured from soy compounds and T biosynthesized by the human body (e.g.-25‰). This arises from differential fixing of $^{12}CO_2$ and $^{13}CO_2$ in soy and related plants.(Krueger HW & Reesman RE, 1982). Thus by determining the $\delta^{13}C$ value of urinary T one can determine its origin. This brief overview greatly over simplifies an exceedingly complex method which is highly dependent on the underlying analytical methodology. If the extraction and chromatography steps are not perfectly executed to provide complete baseline separation of the steroids, the results will be unintelligible.(Aguilera et al. 1999)

Correct interpretation of the $\delta^{13}C$ values requires an understanding of factors that influence them. This study focuses on clinical studies that probe the underlying physiology and pharmacology of the method. It concerns endocrinology, steroid metabolism and pharmacokinetics. In the past seven years we have developed and optimized the IRMS method, (Aguilera et al.1996; Aguilera et al. 1999; Aguilera et al. 2000; Aguilera et al. 2001) determined $\delta^{13}C$ values of various urinary steroids, validated our screening method which determines $\delta^{13}C$ values on androsterone and etiocholanolone (Aguilera et al. 2000) and validated a confirmation method which determines $\delta^{13}C$ values of three urinary diols. (Aguilera et al. 1996; Aguilera et al. 2001). We have also presented evidence that the IRMS method is useful for distinguishing cases of naturally elevated TE(Aguilera et al. 2001) from T users. Others have confirmed (Shackleton et al. 1997a) and extended our studies.(Shackleton et al1997b; Ueki & Okano 1999). Our results show, as expected, that T administration lowers $\delta^{13}C$ values of urine T metabolites. All the previous studies delve into this method, its efficacy, and the $\delta^{13}C$ values of healthy subjects, whereas this study probes the underlying physiology, endocrinology, and pharmacology of the application of the IRMS method.

It is reported (Catlin DH et al; Wang et al.1998) that some individuals do not experience an increase in TE when T or related steroids are administered. The lack of response is often attributed to the Asian ethnic group, although we have found that some non-Asians also do not respond to T. We believe, but have yet to unequivocally prove, (Catlin DH et al.) that the population we study (U.S. students) is bimodal with respect to baseline TE and that this is one determinant of the response to T. The high mode (HM) group is characterized by relatively high baseline T and E excretion rates, whereas the opposite is the case for the low mode (LM) group.

The specific aim was to conduct a pharmacokinetic study of T using $\delta^{13}C$ values, TE, T metabolite concentrations, and other variables on serum and urine samples collected from 12 healthy male volunteers who receive a zero-order infusion (constant dose) of testosterone for six days preceded by a two-day baseline period. This experimental paradigm has been used to determine the responsiveness of the hypothalamic-pituitary-testicular (HPA) axis to FSH and LH. On day 1 and 2 of the T infusion the dose is 7 mg/m^2/24h which is the T production rate of an average male. On infusion days 3 and 4, the T dose is doubled to 14 mg/m^2/24h, and on days 5 and 6 the T infusion is doubled again to 28 mg/m^2/24h. On days 6 and 7 the post infusion decline in TE and $\delta^{13}C$ values is determined. Prior to the infusion, the subjects will receive a test dose of transdermal T that will determine, on the basis of the concentrations of urinary steroids, into which mode they are placed. Six subjects will be in the HM group and 6 in the LM group.

2B. BACKGROUND on PHARMACOKINETICS OF T, E, AND TE

Total urinary T is the sum of urine T glucuronide (TG), T sulfate (TS) and free T. In doping control, the T portion of the TE that is measured is TG (or TG+T) because the samples are deconjugated before the TE is determined. The same is true for urine E. An elevation in urine TE must be the resultant of an increase in plasma TG leading to an increase in urinary TG or a decrease in plasma EG leading to a decrease in urinary EG or both. Thus compared to the LM group, the HM group must experience a greater increase in the excretion rate of TG or a lesser decrease in EG or both. This study will determine which of these three possibilities is correct. (If our hypothesis that HM and LM is a determinant of the urine TE response to infused T proves to be incorrect, the study will still be successful because we do know that there are at least two sub-populations that respond differentially to T, and both will be represented in the study.)

For a greater increase in TG to occur, the clearance of TG into urine must increase in the HM group relative to the LM group. The total clearance (CL) of intravenous T from plasma in a 70 kg man is about 800 L/day. (Ke=0.7, $t_{1/2}$ = 1 h, Vd=50 L). Since urinary free T excretion rates are very low (160 ng/h), the CL of free T from plasma does not significantly involve the kidney (CL <1 L/day). Likewise the renal CL of TG is also very low ~62 L/day (calculated from data in Peng et al., 2002). The low urine CL of T and TG are confirmed by studies showing that less than 1% of a dose of T appears in the urine as T or TG (Baulieu & Mauvais Jarvis, 1964; Leder et al. 2001) Thus most of the CL of T from plasma is metabolic CL by the liver and other tissues, and the majority of this is metabolism to androsterone and etiocholanolone which appear in urine. It follows that the most likely explanation for differences between the HM and LM could arise from differences in renal CL of TG or the metabolic CL to androsterone and etiocholanolone.

The E side of the equation is more complex because the CL of E from plasma has not been fully elucidated. It is known that the production rate (PR) or E is about 1/17th of the PR of T and the urine PR of EG is about 100 ug/day. Two urinary diol metabolites and one putative E precursor have been identified in urine, and there is good evidence that E is linked to the HPA axis.(Dehennin & Matsumoto 1993; Dehennin 1993). High levels of T inhibit E production and low levels of T stimulate E production. In our study we expect to determine if the decline in urine EG is the same in the HM and LM groups. If the decline is less for the LM group, we will have determined one reason why this group is relatively resistant to a rise in TE.

Because the pharmaceutical T infused in this study will have a low $\delta^{13}C$ value, we will be able to determine the urinary kinetics of androsterone and etiocholanolone, even during the replacement dose. This will enable us to determine the time at which the $\delta^{13}C$ values become positive, and provide an estimate of the relative sensitivity of urinary TE and urinary $\delta^{13}C$ values.

3. METHODS:

The clinical trial paradigm was the fundamental method. The infusion was carried out in the UCLA Clinical Research Center (CRC) which is funded by the National Institute of Health (NIH) for this type of study. The subjects were males as described in Table 1. Blood and urine were obtained at appropriate intervals and analyzed for various clinical parameters in the UCLA Hospital Laboratory and by specific methods used in the UCLA Olympic Analytical Laboratory.

The analysis of urinary steroid concentrations was carried out using a modification of a previously reported method.(Breidbach & Catlin 2001) A sample volume of 0.25 mL was used for analysis of highly concentrated steroids. The conditions used for GC/MS analysis have been previously reported.(Breidbach & Catlin 2001) T and E concentrations were calculated using peak height ratios relative to [16,16,17α-$^{2}H_{3}$]testosterone (D3T). Androsterone and etiocholanolone concentrations were calculated using peak area ratios relative to [2,2,3β,4,4-$^{2}H_{5}$]etiocholanolone. All other concentrations were determined using a calibration curve prepared in a matrix of steroid-free urine.

Steroid $\delta^{13}C$ values were measured by GC/C/IRMS using a previously reported method.(Aguilera et al. 1999) All $\delta^{13}C$ values are calculated relative to the Vienna Pee Dee Belenmite reference standard (vPDB).

Serum T concentrations were measured using a previously reported method.(Wang et al. 2004) In brief, D3T was added to 2 mL serum as intenal standard and the serum was extracted with diethylether. After evaporation of solvent, steroids were reconstituted in 100uL methanol and 15uL was injected into the LC-MS-MS. T concentrations were calculated using a calibration curve prepared in a matrix of newborn calf serum.

Testosterone excretion rates expressed as micrograms per day for the individual subjects were analyzed by mixed-effects linear models (SAS Proc Mixed) to estimate group means and test for significant effects of mode, day and dose. Student's T-test was used to test for significant differences between HM and LM baseline excretion rates.

Subjects: Twelve healthy males were recruited as planned. They volunteered to participate in a CRC study designed to characterize the effects of intravenous testosterone on the hypothalamic-pituitary-testicular axis and the excretion of testosterone and its metabolites. The study was approved by the UCLA IRB (#02-02-056-03) and all subjects gave written informed consent. A medical screening questionnaire was completed and subjects were free from any medical problems and had no illnesses affecting their kidneys, liver or reproductive hormones. None of the subjects were taking any prescription medications or nutritional supplements. The subjects were categorized as either low mode (LM, N= 6) or high mode (HM, N = 6) by their urinary testosterone to epitestosterone ratios (TE). The demographics of the subjects are presented in Table 1.

Protocol: After collection of the baseline urines (day 1), the subjects received a continuous intravenous infusion of testosterone. The original protocol called for a six day infusion with two days at 7 mg/m^2/24-h, two days at 14 mg/m^2/24-h, and two-days at 28 mg/m^2/24-h. Three unexpected issues intervened: one, the FDA requirements were quite onerous, two, subjects were very difficult to recruit, and three, the literature on T infusions that we relied on was faulty, in fact T was not soluble at the high dose. Accordingly we adjusted the protocol. The length of infusion at each dose was reduced from 48 to 24 hours, and the dose on day 2 was 7 mg/m^2/24-h (the average daily production rate of testosterone in the normal male) on day 3 the dose was doubled to 14 mg/m^2/24-h and on day 4 it was increased again to either 21 or 28 mg/m^2/24-h. After cessation of infusion, urine and serum collections continued for two days.

Blood was drawn once per day in 24-hour intervals such that collections took place prior to beginning infusion on days 2, 3 and 4. All urine was collected on each experimental day in timed 4 and 8-hour periods: 08:00-12:00, 12:00-16:00, 16:00-20:00, and 20:00-08:00. The volume of urine was recorded and several 10-ml aliquots were saved at -20°C for subsequent analysis.

4. RESULTS:

A. Serum testosterone

Mean serum T concentrations by day are presented for LM and HM subjects in Figures 1 and 2, respectively. Serum was not available for LC-MS-MS analysis for day 6 in subjects 1,4, 12 and 17 for day 5 in subject 21. The general pattern observed for both the LM and HM groups is a successive increase in serum T as the dose is increased on days 2, 3 and 4. The average baseline concentration for both groups is approximately 500 ng/dL. Although concentrations do vary widely from subject to subject, average concentrations for both groups increase to approximately 1000, 1500 and 2000 ng/dL on days 2, 3 and 4, respectively. On day 5 after infusion has ceased, serum T drops below baseline levels. On day 6, although levels increase relative to day 5 for all subjects measured, the levels remain below baseline.

B. Steroid excretion rates

Mean urinary steroid excretion rates expressed as micrograms per day and percent change from baseline are presented by group in Table 2 below. The excretion rates of all steroids analyzed increased during the first day of the infusion (day 2) and continued to climb in response to increasing rates of infusion over the course of days 3 and 4. Mean urinary steroid excretion rates of T, E, androsterone, etiocholanolone, 5α and 5β-diol expressed as micrograms per hour over 12-hour intervals for subject 10 from the LM group are presented in Figure 3 below where a pattern of increasing excretion rates in response to increasing rates of infusion can be clearly seen. Mean urinary T excretion rate expressed as micrograms per hour over 12-hour intervals for all subjects is presented in Figure 4 below. A clear contrast can be seen between groups with maximal rates of excretion ranging between 1 to 4μg/h for 5 out of 6

subjects in the LM group while the for the HM group maximal rates ranged from 20 to 50μg/h. Subject 12 from the LM group differed from all others in the group in that during the highest rate of infusion his T excretion rate reached 25μg/h. Mean urinary T excretion rates expressed as micrograms per day for the LM and HM groups are presented in Figure 5. Urinary T excretion rates are clearly seen to be increasing in response to increasing rates of infusion for both groups. It is not until day 4 at the highest rate of infusion that the mean excretion rate for the LM group exceeds the baseline rate of the HM group.

Analysis of the effect of doses of 21or 28 mg/m^2/24 h on the excretion rate of T showed no significant difference ($p=0.2015$). The difference in effect of a dose of 21or 28 mg/m^2/24 h remained insignificant when taken in conjunction with HM ($p=0.7314$) or LM ($p=0.8400$) groups. Therefore in all statistical analysis of T excretion rate, treatment day was used in place of dose.

Within the HM group, day 2 excretion rates did not differ significantly from day 1 ($p=0.19$) but all other differences between days 1 through 4 were significant ($p<0.01$). Within the LM group only days 1 and 4 differed significantly ($p=0.04$). In comparison of the response between groups, baseline excretion rates were higher in the HM group than the LM group ($p=0.0049$). During infusion, excretion rates on days 3 and 4 were significantly higher in the HM group than in the LM group ($p<0.001$). P-values for between group comparisons on days 1 through 4 are presented in Table 3 below.

Mean urinary E excretion rates expressed as micrograms per day for the LM and HM groups are presented in Figure 6. The mean baseline excretion rate of E for the LM group is approximately 50% higher than that of the HM group, however, as E excretion increases during T infusion the difference in rates between the two groups decreases until on day 4 at the highest rate of infusion when the excretion rate of the HM group exceeds that of the LM group by approximately 25%. On day 5, upon cessation of the infusion, E excretion rates decline for both the LM and HM groups with mean excretion rates for the LM group falling below baseline levels. Excretion rates continue to decline for both groups on day 6 with both groups below baseline levels.

Mean TE ratios by day are presented below for LM and HM subjects in Figures 7 and 8, respectively. Only one subject from the LM group exceed a mean TE of 4 and only while T was being infused at the highest dose. One subject in the HM group did not exceed the threshold on any day. While all others in the group were above the threshold throughout the infusion Subjects 7 and 16 in the HM group had mean baseline TE of 4.8 and 6.7 and therefore did not cross thresholds of 4 and 4 or 6, respectively during the administration period.

We had hypothesized that urine TE would exceed 6 on day 3 or 4 for the HM group. Subjects 5, 7 and 16 had mean TEs in excess of 6 on day 2 of the study which was earlier than we had predicted. However, for subject 4 mean TE did not exceed 6 until day 4. In the case of subject 3, the mean TE did not exceed 6 on any day. The results for the LM group were much as we had predicted where daily mean TE did not exceed 6 on any day. Within the LM group,

subject 12 is singular in having exceeded a mean daily TE of 4. Although it cannot be seen in figure 5, subject 12 did in fact exceed a TE of 6 several times on day 4, however, at other times during day 4 the TE was less than 1. No other subject responded to the infusion in this way.

C. Rates of incorporation of ^{13}C into steroid metabolites

The testosterone used for the infusion was found to have a $\delta^{13}C$ value of -29.3 $\pm 0.1^{0}/_{00}$. Epitestosterone was not detected in the material. Daily mean $\delta^{13}C$ values for androsterone, etiocholanolone, 5α and 5β-diols for LM and HM groups are presented in Figures 9 and 10, respectively. In all cases, the $\delta^{13}C$ values become more negative with increasing dose and then return to an intermediate value on day 5 after infusion has stopped. By day 6, the $\delta^{13}C$ values have approached but not quite reached baseline levels. In the cases of androsterone and etiocholanolone, the observed patterns for the LM and HM groups are strikingly similar with etiocholanolone $\delta^{13}C$ values being 0.5- 1.0 more negative than those of androsterone. With respect to the 5α and 5β-diols, the patterns observed for the LM and HM groups are also quite similar, however, the $\delta^{13}C$ value for the 5α-diol appears to have reached a limit on day 3 in the case of the LM group; something which does not occur for the HM group until the following day. It can also be seen that on day 5 after the infusion has been terminated, the $\delta^{13}C$ value for the 5α-diol has returned to an intermediate level for the HM group but not the LM group.

Mean $\delta^{13}C$ values and excretion rates for 1 subject each from the LM and HM groups are presented in Figure 11 below. Only those collections having sufficient E concentration to allow measurement of $\delta^{13}C$ values are represented in this figure. Both subjects show an increase in the rate of E excretion during infusion and the increasingly negative $\delta^{13}C$ values indicate that E originates from infused T.

D. Threshold TE:

Collection intervals (hours) from the beginning of infusion at which TE exceeds 4 and 6 and collection intervals (hours) from the end of the infusion until the TE falls below 4 and 6 are presented for individual subjects by group in Table 4 below. Instances of "Never" and "Always" for the infusion and post-infusion intervals, respectively, indicate that a subject's TE did not exceed a given threshold during the infusion. Using LM subject 12 as an example, it can be seen that during the collection interval beginning at 58 hours after infusion and ending 1 hour later, the TE exceeded both 4 and 6. Continuing with subject 12, it can be seen that during the first 4-hour collection interval after infusion ceased the TE dropped below both thresholds. Although subjects 7 and 16 in the HM group had baseline TE above 4 and 6, respectively, after infusion ceased their TE fell below 4 and 6 during the indicated interval.

E. Threshold $\delta^{13}C$ values:

We have previously established threshold $\delta^{13}C$ values of −25.45, −23.97, −28.46 and −28.39 for etiocholanolone, androsterone, 5β-diol and 5α-diol, respectively and threshold differences between 5β-diol and 5α-diol and 5β-pdiol (endogenous reference compound, ERC)

of 3.473 and 3.986, respectively. These $\delta^{13}C$ values and differences correspond to 3SD units beyond the means for our male control population. Collection intervals (hours) from the beginning of infusion at which etiocholanolone, androsterone, 5β-diol and 5α-diol $\delta^{13}C$ values fall below their respective thresholds and the collection intervals (hours) from the end of the infusion until $\delta^{13}C$ values rise above the same thresholds are presented for individual subjects by group in Tables 5 and 6 below. It can be seen in Table 6 that for HM subjects 5 and 7, the difference between the 5α-diol and ERC remained outside the threshold in the final collection interval.

F. Relative ability of the IRMS and TE methods to detect T administration:

Only one LM subject exceeded a threshold TE of 4. This subject exceeded a threshold TE of 6 and only after the rate of infusion was increased to 14 mg/m^2/24-h and within the first 4-hour collection interval following cessation of infusion, his TE was already <4.

In contrast, 4 of 5 HM subjects with baseline TE less than 6 had exceeded that threshold within 33 hours of the initiation of infusion. Most of the HM subjects exceeded a threshold of 4 during the first 4-hour collection interval after infusion had begun. For HM subjects on average the maximum elapsed times to return below threshold TE of 6 and 4 were 7 and 11 hours, respectively. Discounting those subjects with baseline TE in excess of the threshold, the maximum time required to return below a threshold TE of 4 was 21 hours.

Using IRMS methodology and in particular the differences between both the 5α and 5β-diol and the endogenous reference compound (ERC), all subjects would exceed 3SD relative to the mean of our established normal population within 36 hours. More time and higher rates of infusion were required for the $\delta^{13}C$ values of the 5β-diol to exceed population limits. While 1 LM subject returned to the normal range during the first collection after cessation of infusion, the minimum times required for both differences relative to ERC to return to within normal range were ~13 and 27 hours for the LM and HM groups, respectively.

A total of 274 urine samples (HM=120, LM=154) were collected during the time periods when T infusion was taking place. Of these, 174 (HM = 27, LM = 147) had TE ratios less than 4 and would therefore not generate an adverse analytical finding by the TE method alone. When these same urine samples were analyzed using the IRMS method, 138 (HM=24, LM=114) of the 174 samples with TE ratios less than 4 satisfied our criteria for an adverse analytical finding (differences between both the 5α and 5β-diol and the ERC in excess of 3SD relative to the mean of our established normal population).

The TE method thus has a differential ability, dependent upon baseline TE to detect T administration. No such differential ability is apparent using IRMS methodology. The relative strengths of the TE and IRMS methods are most obvious within the LM group where even at rates of T infusion of 28 mg/m^2/24-h a threshold TE of 4 was not exceeded for most individuals while $\delta^{13}C$ differences relative to ERC remained outside 3SD limits for as much as a day.

G. Pharmacokinetic explanation for the differential response of the HM and LM groups to T:

The recent publication by Jakobsson et al. 2006 is likely relevant to the differences between HM and LM subjects although the exact relationship to the present study is not clear. These authors described a bimodal population in terms of secondary metabolism of steroids.

5. DISCUSSION AND CONCLUSIONS:

Previous studies have shown that T administration results in a decrease in E excretion. We note that the results of one study showed that labeled E was recovered after oral administration of dideuterium labeled T (20 mg). In that study, slightly more than 50% of the labeled material could be accounted for by measuring selected urinary steroids. Labeled E accounted for approximately 0.1% of the recovered label. Elucidation of the underlying mechanism is warranted and would require further study.

In our previous T administration study the protocol differed in that we maintained the infusion rate for 2 days before increasing it again. Therefore one possible way to reconcile the findings of these two studies would be to invoke a delay in the suppression of E production. This coupled with the fact that our previous study did not show E excretion rates falling below baseline levels until the 4th day (2nd day at 14 mg/m^2/day rate of infusion), we might have predicted that suppression of E production would not be registered until the 4th day of the current study (coinciding with cessation of infusion). In fact we do see that E excretion rates are below baseline levels by day 6 of this study.

6. FUTURE PLANS:

We would like to reapply for a new grant to continue the work. The number of startling new findings were not expected and really should be pursued as they are highly relevant to doping control. On the other hand the clinical aspects of the study (FDA, CRC, Human Subjects) proved to be far more difficult than expected and led to cost-overuns, thus to continue would take a substantial increase in funding. Now that it is complete, the study might be considered a pilot study. Indeed it has opened up several avenues for future pursuit.

7. EXPENDITURES:

The difficulties with obtaining and retaining subjects, obtaining approval from the U.S. Food and Drug Administration, and the T solubility issue led to substantial time delays and to substantial cost over-runs. The total award amount was $183,000.00 and our total expenditure from July 1, 2003- June 30, 2005 was $182,994.16.

The breakdown is as follows:

Salaries- Academic	$38,824.00
Salaries- Staff	$45,372.25
Salaries- General Assistance	$26,127.02
Employee Benefits	$24,439.71
Supplies & Expenses	$17,732.15
Overhead	$30,499.03
TOTAL	**$182,994.16**

8. PUBLICATIONS:

Many of the finding warrant publications in peer-reviewed journals. This is our intention.

9. APPLICATION TO DOPING CONTROL AND ADVANTAGES OVER EXISTING METHODS:

The main implication for doping control is that T administration by athletes with low TE that we have tentatively characterized as LM subjects will be difficult to detect by conventional doping control methods that rely on screening TE. These subjects are readily detected if the carbon isotope ratio method is used. The infusion of high doses of T to most LM subjects does not lead to an elevation in urine TE.

The ability of the TE to detect T users in HM subjects is confirmed and further characterized. The duration of elevated TE ratios occurs earlier and lasts longer when T is administered by a zero-order IV infusion.

The findings presented herein together with the known ability of the carbon isotope ratio method to detect users of endogenous steroids other than T, for example DHEA and androstenedione, argues persuasively for greater use of the method in conventional doping control.

10. ONE PAGE SUMMARY FOR WADA WEBSITE

We investigated the hypothesis that a population of males might exist that could dope with testosterone and yet not be detected by the conventional TE that is used in doping control to detect testosterone users.

Twelve healthy male subjects were stratified as high mode TE (HM) or low mode (LM) based on their resting baseline urine TE. All received a three-day continuous intravenous infusion of testosterone. Serum and urine were collected for one day prior to the testosterone

infusion, during the infusion, and for two days following the infusion.

The main hypotheses were that subjects with low TEs would be difficult to detect by the conventional TE screen used in doping control and that the carbon isotope ratio method would be a superior method for the conventional screening method. The hypothesis proved to be correct.

The excretion rates of all steroids increased during the first day of the infusion when the dose was equivalent to the normal production rate of testosterone. When the dose was increased to double and three times the normal production rate, the excretion rates increased still more. While the rates increased for both the HM and LM groups, in the case of the HM subjects the increases occurred earlier and were higher. In fact 5 of the 6 LM subjects never achieved a TE greater than 4 while the TEs exceeded 4 in all the HM subjects.

Based on other studies we expected the testosterone infusions would lower the concentration of urinary epitestosterone, however the opposite was found: the epitestosterone levels increased. This unexpected finding raises a number of intriguing questions about the control mechanisms for epitestosterone.

This study provides convincing evidence that all males do not metabolize testosterone in the same fashion and that at least two subgroups exist. Those with low TEs have low testosterone excretion rates and in general are not able to increase the rate above 5μg/h. These subjects are, in effect, protected from detection by the conventional doping control TE method. Further studies are needed to define these groups.

The carbon isotope ratio method detects all testosterone users in both the HM and LM groups and is therefore superior to the conventional TE method. In addition the carbon isotope ratio method is known to detect the use of other doping agents that are difficult to detect by the conventional TE method.

11. REFERENCES

Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG. Improved method of detection of testosterone abuse by gas chromatography/combustion isotope ratio mass spectrometry analysis of urinary steroids. J Mass Spectrometry. 1996;31:169-176.

Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C. Swerdloff RS, Pope HG & Hatton CK. Screening Urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two and androstanediols. J Chromatogr B. 1999;727:95-105.

Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary androsterone and etiocholanolone delta 13 C ‰ values by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Comm Mass Spectrom. 2000;14:2294-2299.

Aguilera R, Chapman TE, Starcevic B, Hatton CK, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with testosterone based on urine diols: controls and athletes with elevated testosterone/epitestosterone ratios. Clin Chem 2001;47:292-300.

Baulieu EE & Mauvis-Jarvis P. Studies on testosterone metabolism. Conversion of testosterone-17-α-3h to 5-α. J. Biol Chem. 1964;239:1569-1577.

Breidbach A, Catlin DH. RSR3, a potential athletic performance enhancement agent: detection in urine by gas chromatography/mass spectrometry. Rapid Comm Mass Spectrom. 2001;15:2379-2382.

Catlin DH, Starcevic B, and Hatton CK. Urinary testosterone and epitestosterone concentrations are bimodal: implications for detecting testosterone administration (In preparation).

Dehennin L. Secretion by the human testis of epitestosterone, with its sulfoconjugate and precursor androgen 5-androstene-3 beta,l7 alpha-diol. J. Steroid Biochem Mol. Biol 1993;44:171-177.

Dehennin L, Matsumoto AM. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. J Steroid Biochem Mol Bio. 1993;44:179-189.

Jakobsson J, Ekstrom L, Inotsume N, Garle M, Lorentzon M, Ohlsson C, Roh HK, Carlstrom K, Rane A. Large differences in testosterone excretion in Korean and Swedish men are strongly associated with a UDP-glucuronosyl transferase 2B17 polymorphism. J Clin Endocrinol Metab. 2006;91:687-693.

Krueger HW, Reesman RH. Carbon Isotope analyses in food technology. Mass Spectrometry Reviews. 1982;1:205-236.

Leder BZ, Catlin DH, Longcope C, Ahrens B, Schoenfeld DA, Finkelstein JS. Metabolism of orally administered androstenedione in young men. J Clin Endocrinol Metab 2001;86:3654-3658.

Peng SH, Segura J, Farre M, Gonzalez JC, de la Torre X. Plasma and urinary markers of oral testosterone undecanoate misuse. Steroids 2002;67:39-50.

Shackleton C, Phillips A, Chang T, Li Y. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids. 1997;62:379-387.

Shackleton CH, Roitman E, Phillips A, Chang T. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: potential use in gas chromatography isotope ratio mass spectrometry. Steroids 1997;62:665-673.

Ueki M, Okano M. Analysis of exogenous dehydroepiandrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun. Mass. Spectrom. 1999;13:2237-2243.

Wang C, Berman NG, Veldhnis JD, Der T, McDonald V, Steiner B, Swerdlof RS. Graded testosterone infusions distinguish gonadotropin negative-feedback responsiveness in Asian and white men-a Clinical Research Center study. J Clin Endocrinol Metab 1998;83:870-876.

Wang C, Catlin DH, Starcevic B, Leung A, DiStefano E, Lucas C, Hull L, Swerdloff RS. Testosterone metabolic clearance and production rates determined by stable isotope dilution/tandem mass spectrometry in normal men: influence of ethnicity and age. J. Clin Endocrinol Metab 2004;89:2936-2941.

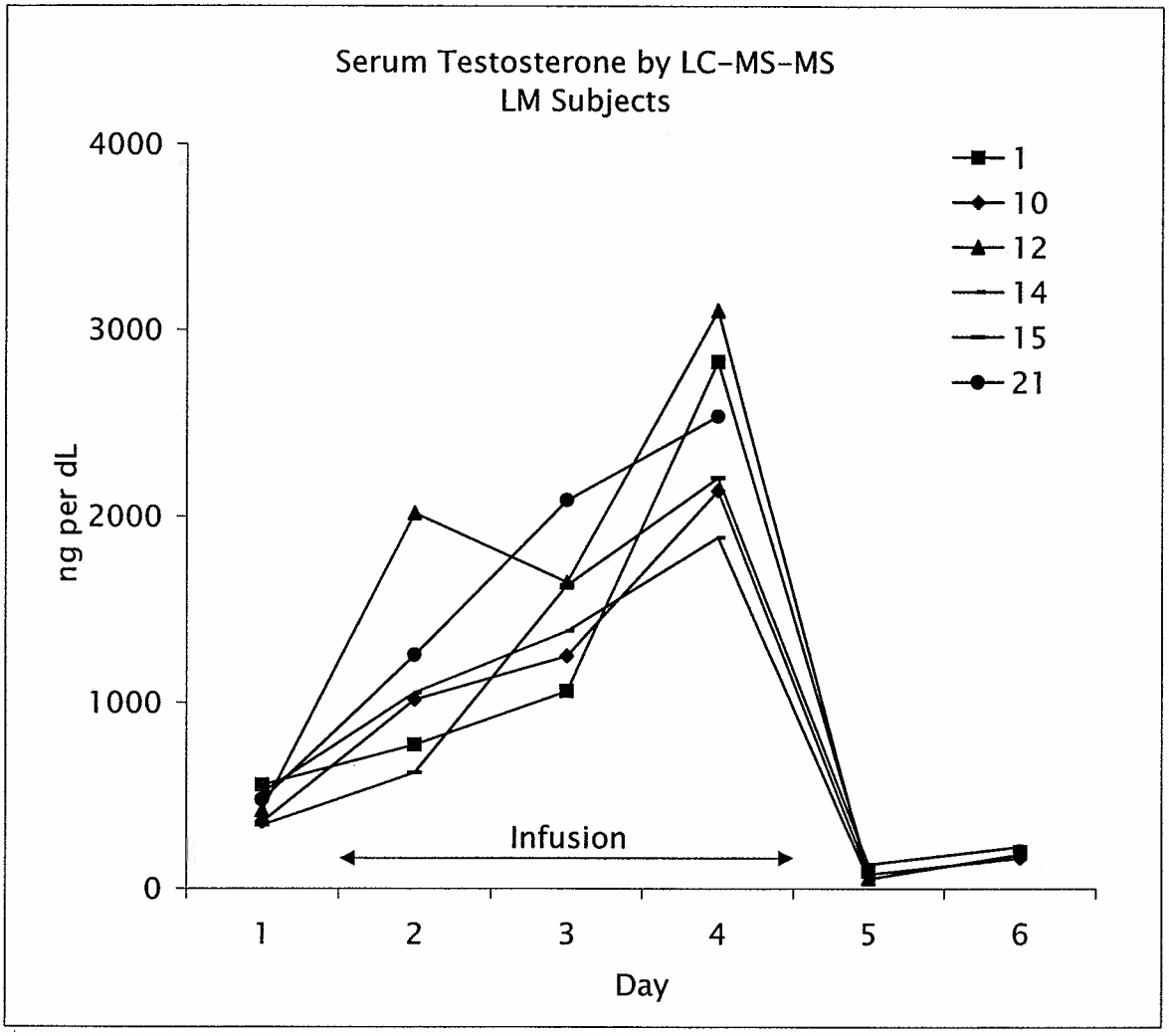


Figure 1. Serum testosterone concentration by day for LM subjects.

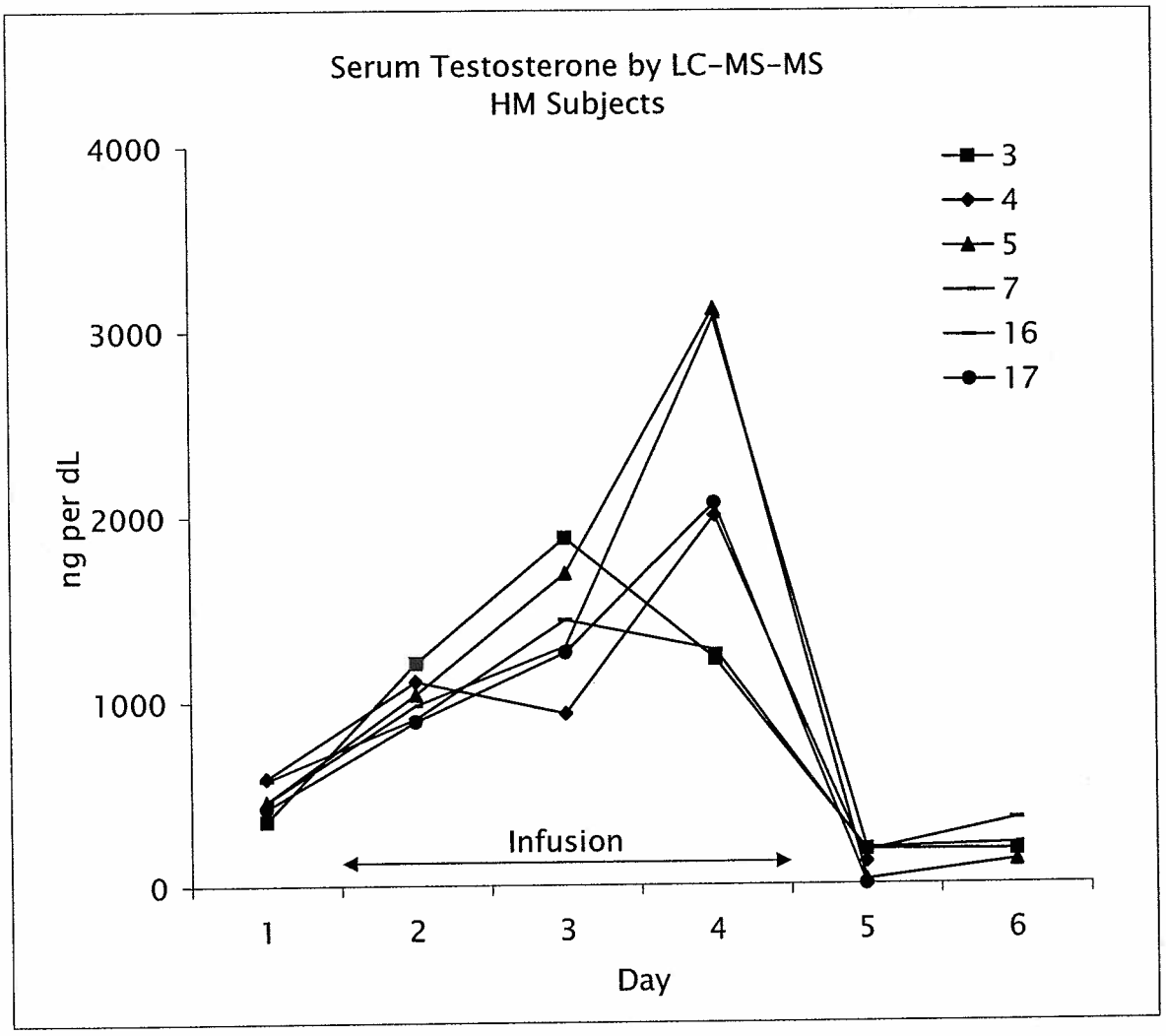


Figure 2. Serum testosterone concentration by day for HM subjects.

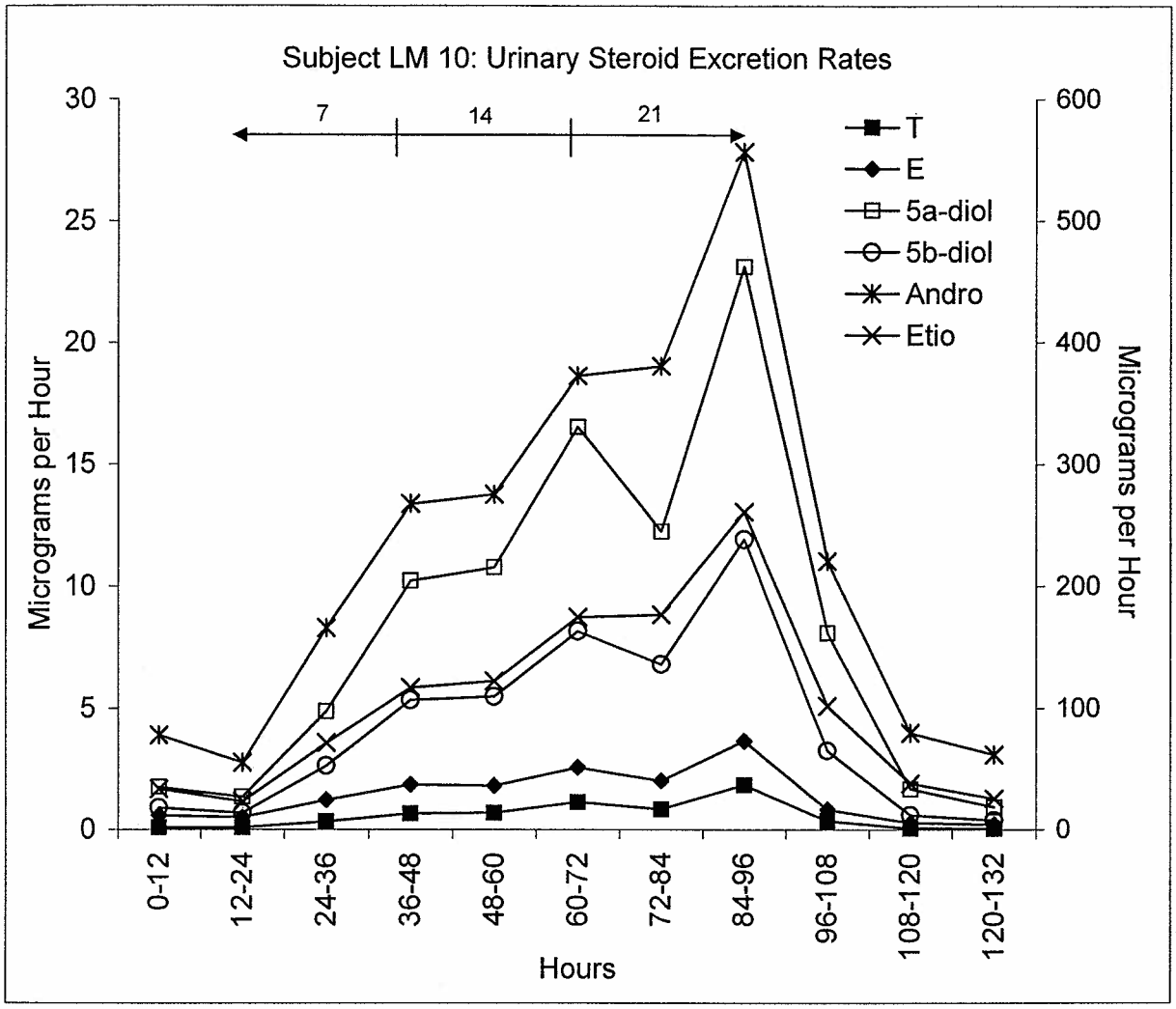


Figure 3. Mean urinary steroid excretion rates over 12-hour intervals for subject LM 10. Rates for T, E, 5α–diol and 5β-diol are plotted on the left axis. Rates for andro and etio are plotted on the right axis. Infusion periods and their respective dosages are indicated along the top of the figure in $mg/m^2/24$-h.

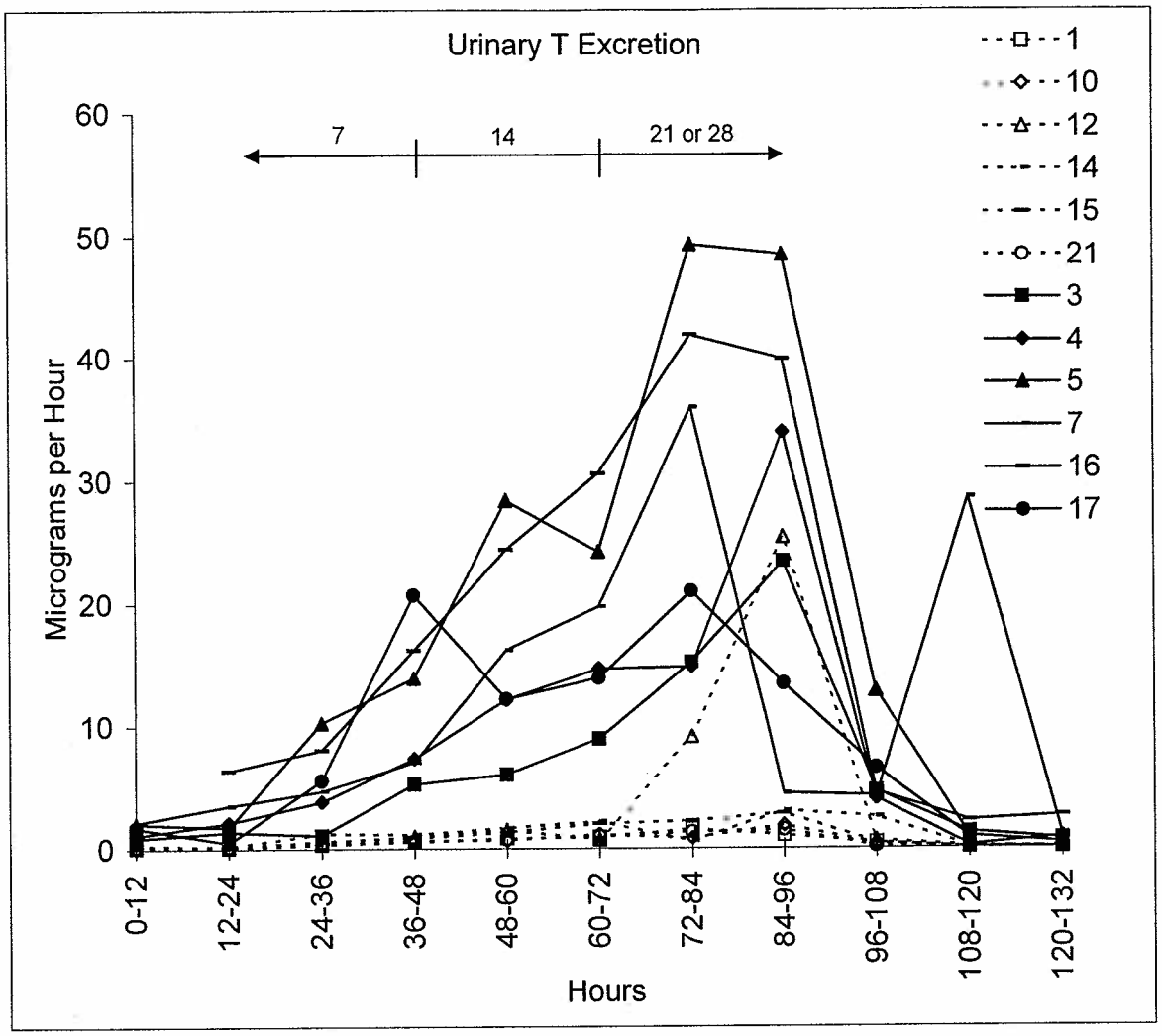


Figure 4. Mean urinary T excretion rate by 12-hour intervals for all subjects. Dashed and solid lines represent LM and HM subjects, respectively. Infusion periods and their respective dosages are indicated along the top of the figure in $mg/m^2/24$-h. Subjects 1, 3, 4, 5, and 7 each received 28 $mg/m^2/24$-h on the final day of infusion.

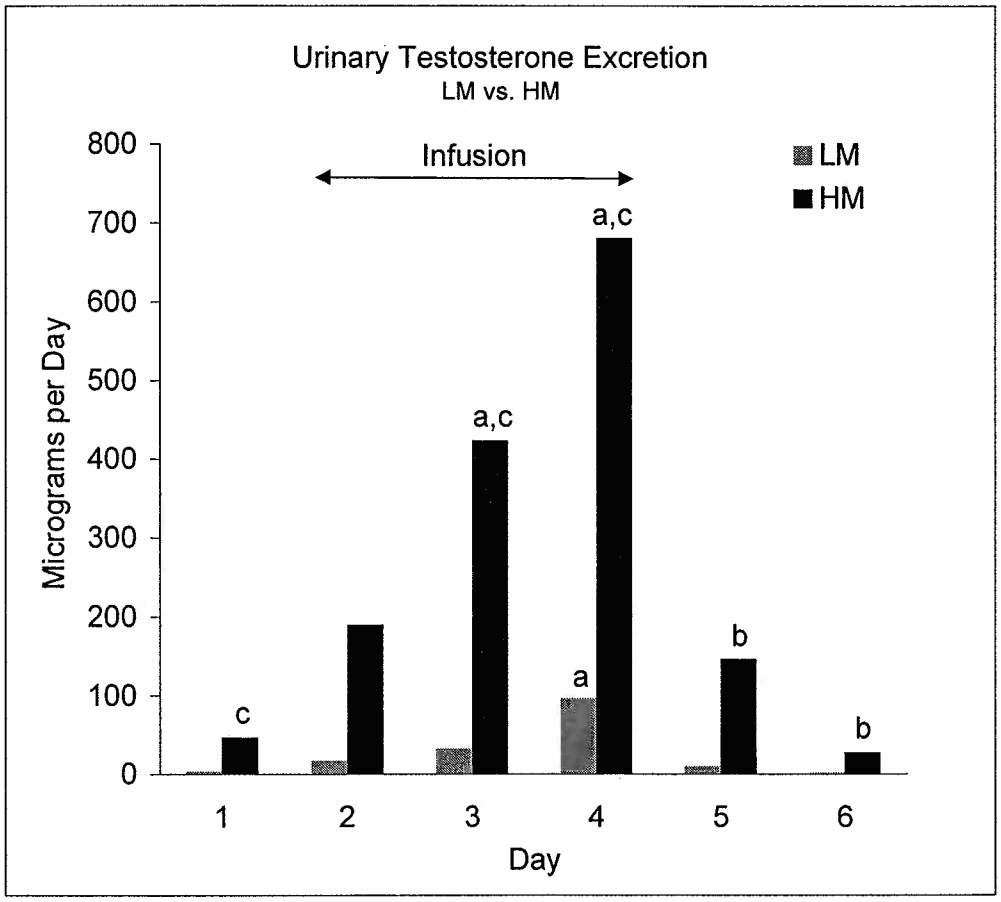


Figure 5. Mean urinary T excretion rates by day and group. a, $P < 0.05$ when compared with day 1; b, $P < 0.05$ when compared with day 4; c, $P < 0.01$ when compared to LM group.

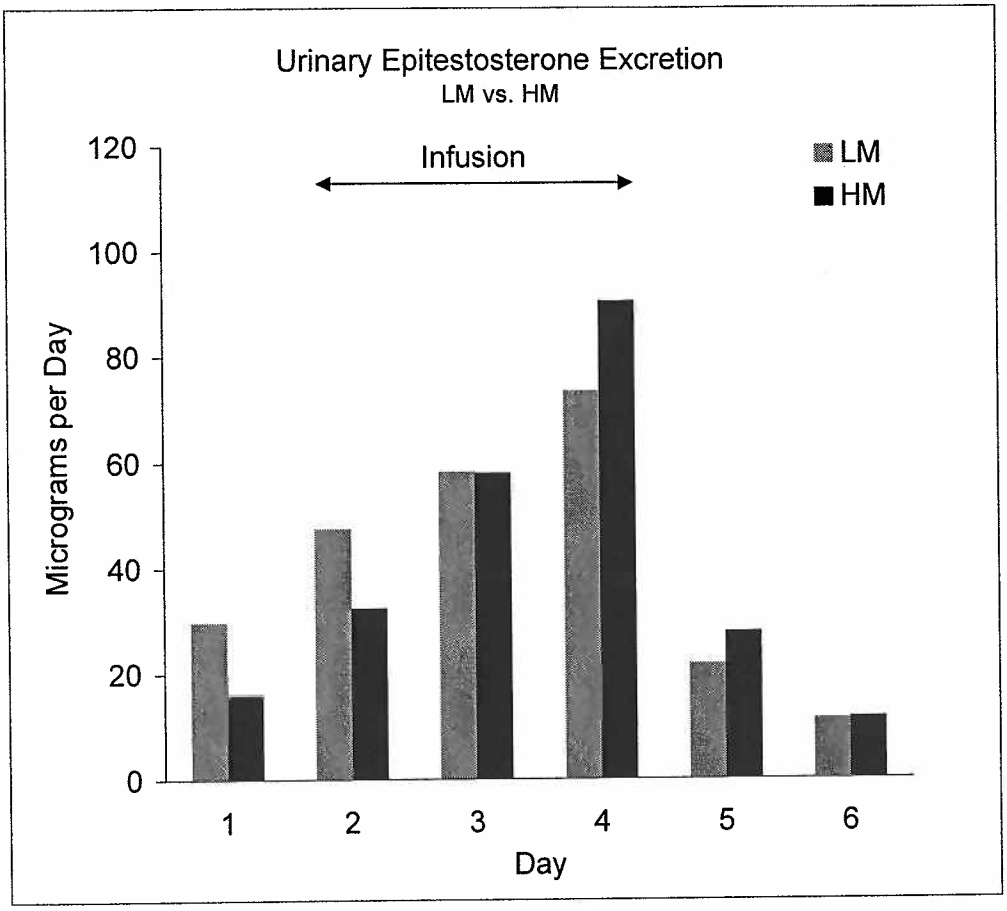


Figure 6. Mean urinary E excretion rates by day and group.

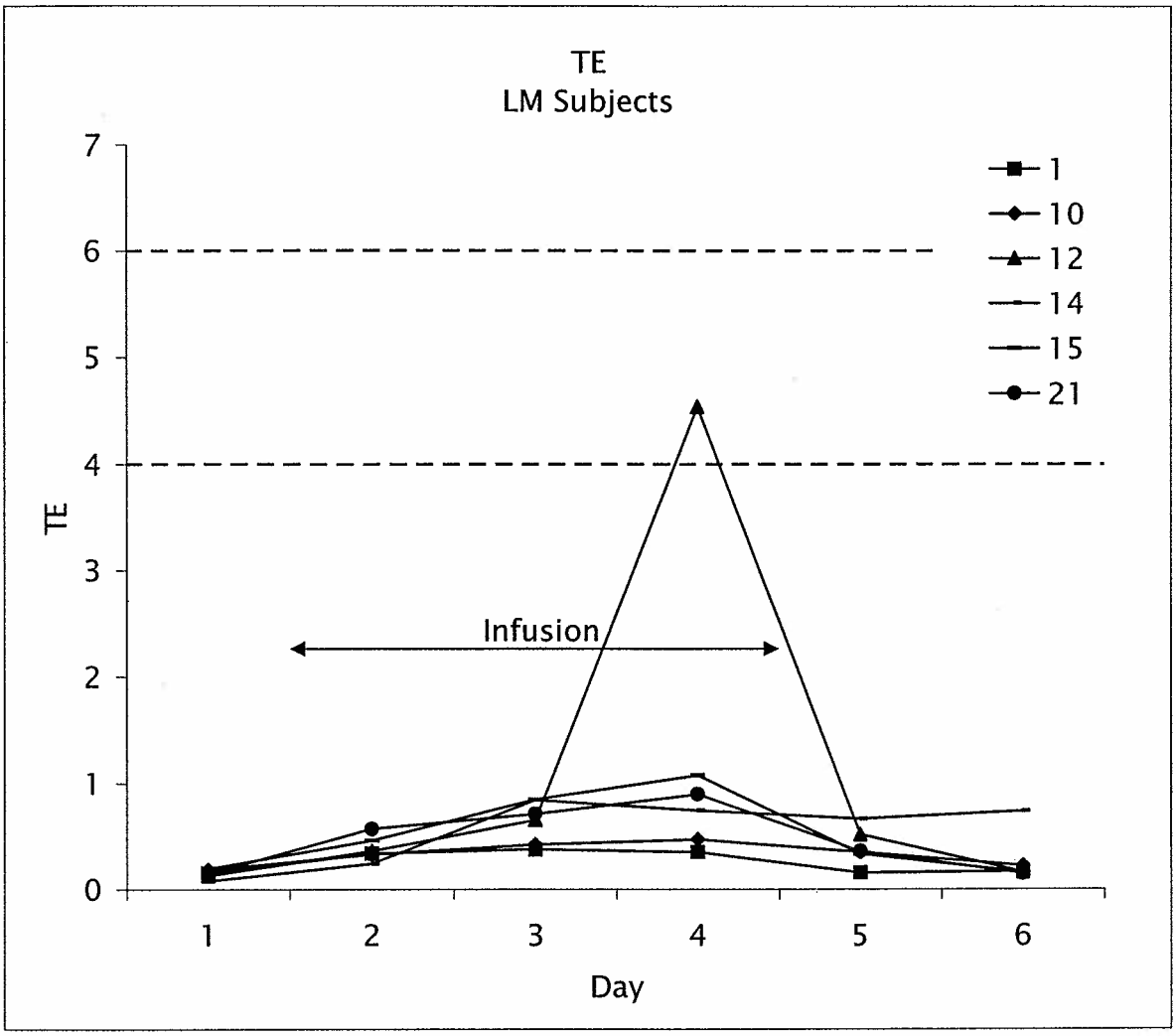


Figure 7. Mean TE for LM subjects by day.

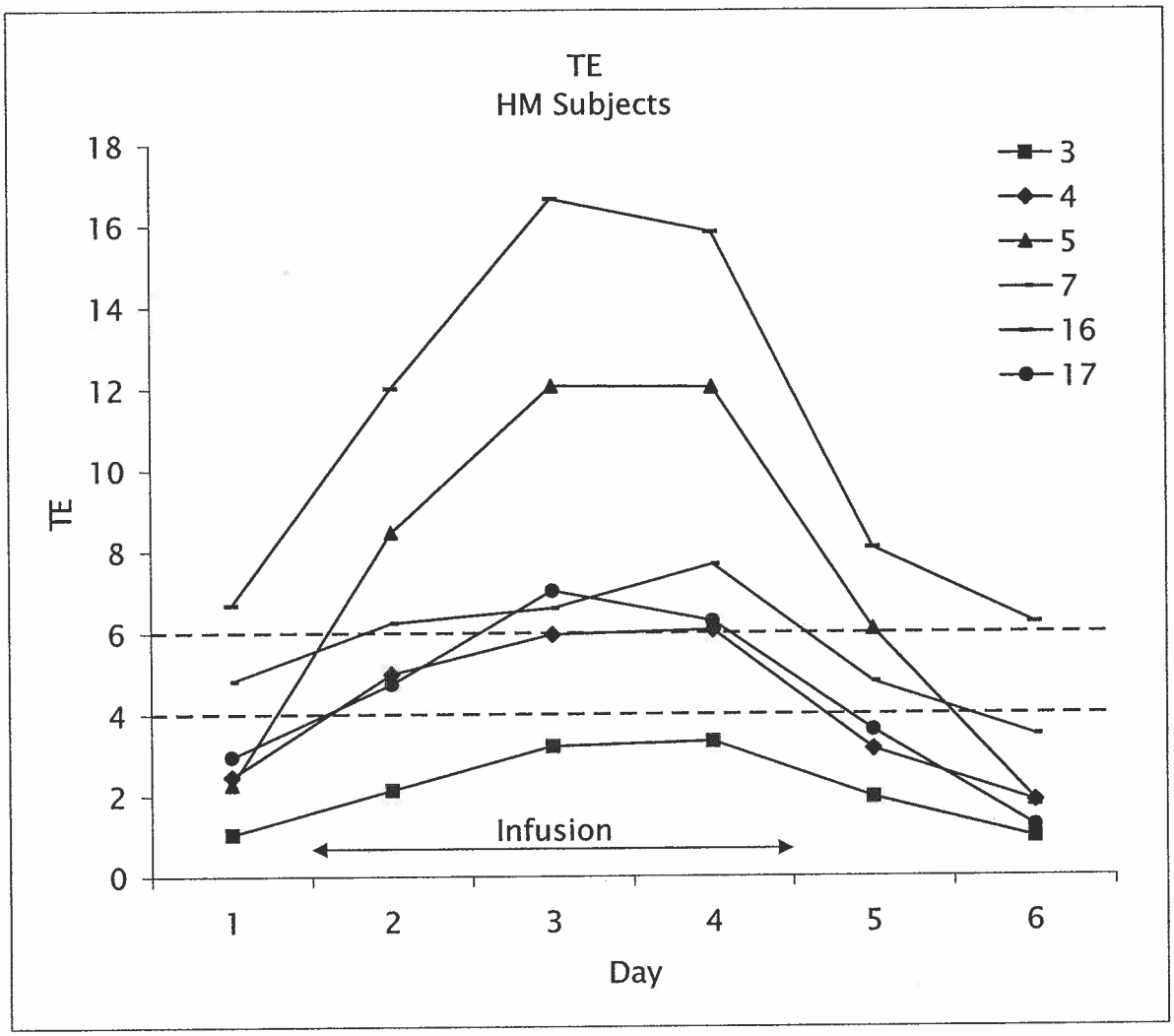


Figure 8. Mean TE for HM subjects by day.

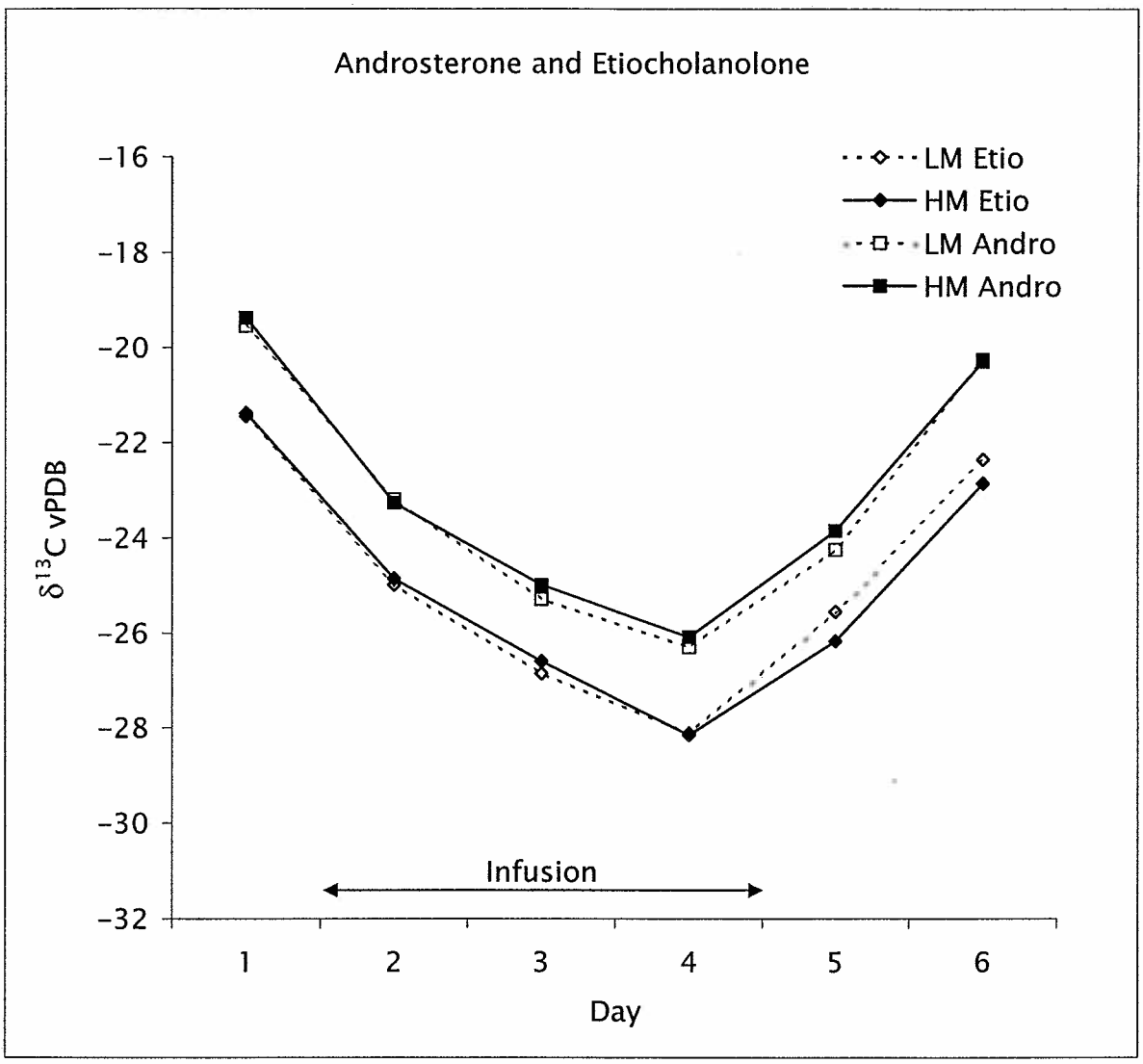


Figure 9. Mean androsterone and etiocholanolone $\delta^{13}C$ values by day and group.

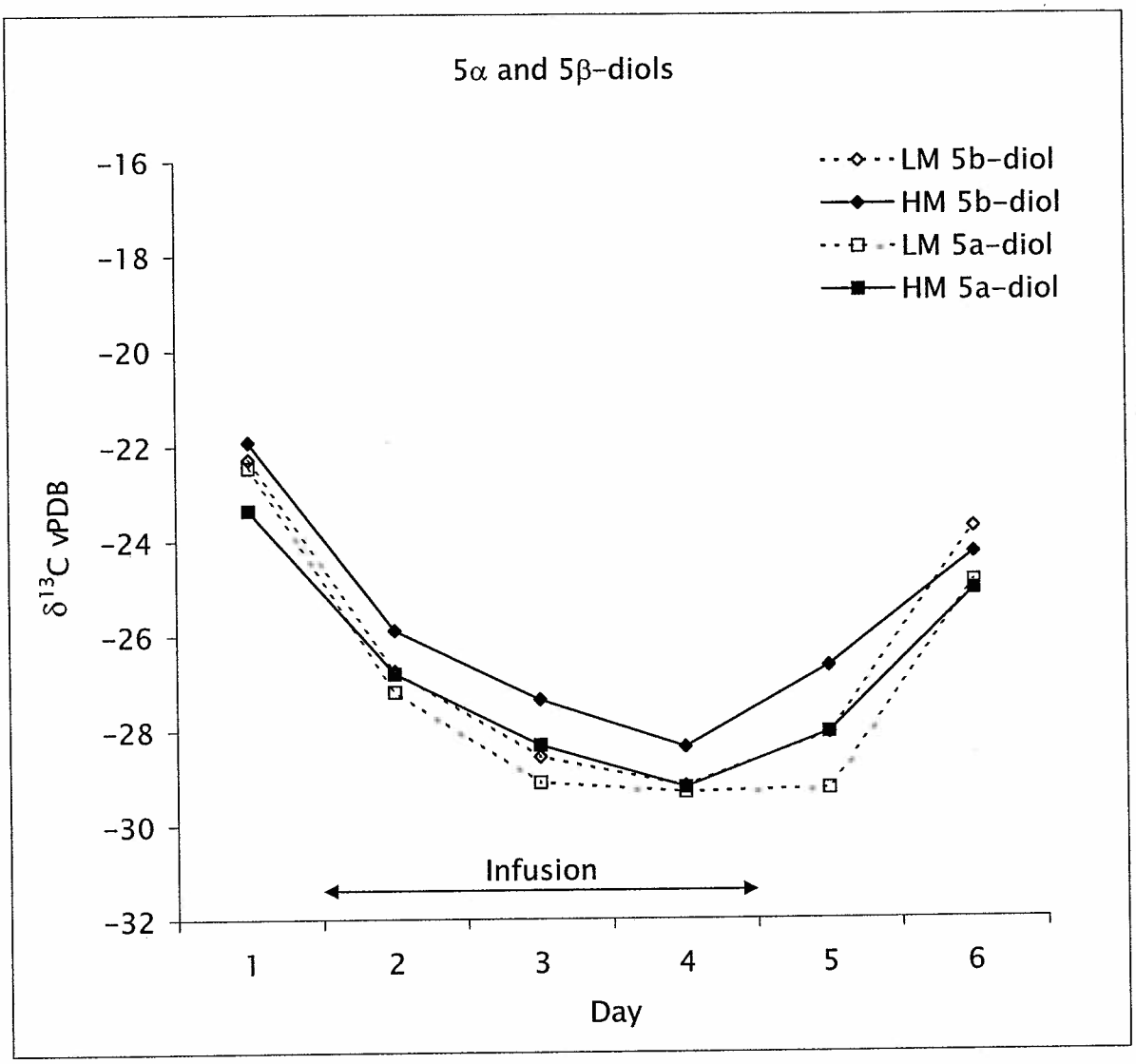


Figure 10. Mean 5α and 5β-diol $\delta^{13}C$ values by day and group.

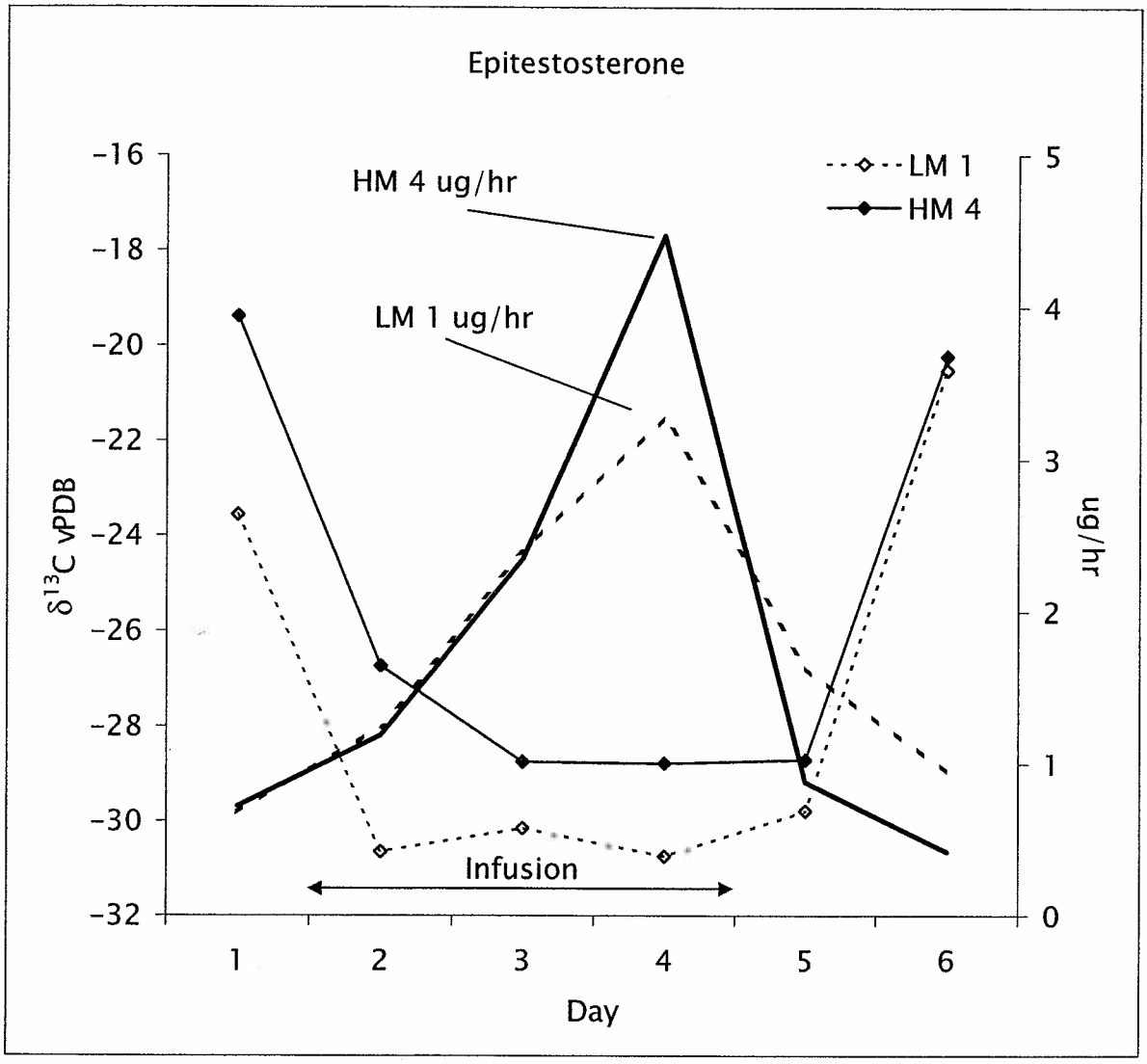


Figure 11. Mean epitestosterone $\delta^{13}C$ values and excretion rates by day for LM subject 1 and HM subject 4.

Demographics of the Study Subjects

	Subject	TE*	Age	BSA (m^2)	Height (cm)	Weight (kg)	Testosterone (ng/dL)	Hgb (g/dL)	FSH (mIU/mL)	Cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
LM	1	0.11	22	1.9	180	74	456	15.2	4.4	194	45	134
	10	0.18	27	1.7	170	61	247	14.3	6.2	179	49	93
	12	0.11	25	2.3	191	98	271	16.5	3.7	137	47	43
	14	0.08	20	2.5	191	116		14.1	1.6	150	60	82
	15	0.13	20	1.8	165	72	307	16.8	2.9	162	58	71
	21	0.15	20	1.7	166	59		15.1	6.5	173	43	108
HM	3	1.8	35	2.0	171	81	521	15.9	0.9	193	49	141
	4	2.1	20	2.0	183	81	379	15	2.7	146	51	88
	5	1.5	41	2.2	182	93	241	13.5	16.1	184	49	123
	7	5.1	25	2.2	191	88		15.7	2.9	179	63	101
	16	4.5	27	1.8	168	67			3.8	125	30	82
	17	2.6	24	1.8	168	70	385	15.5		179	51	118

Table 1. Demographics of the study subjects. *, HM and LM groups differed only with respect to urinary TE ($P < 0.01$).

Urinary Steroid Excretion Rates

		Day (* Infused)										
		1	2*		3*		4*		5		6	
LM	Andro	2580	6170	154%	9800	309%	12900	414%	4540	96%	1980	-21%
	Etio	2080	4110	143%	6520	289%	9660	381%	3160	89%	1440	-24%
	5en3b17a	72	73	3%	60	-19%	49	-25%	44	-37%	40	-37%
	5a3a17a	4	4	11%	5	53%	5	60%	4	1%	3	-25%
	5b3a17a	18	16	41%	19	107%	22	158%	20	35%	13	-24%
	Epit	30	48	81%	58	146%	73	230%	22	-13%	11	-52%
	T	3	17	416%	32	878%	96	2130%	10	174%	2	-51%
	5a3a17b	103	240	155%	439	361%	489	476%	238	119%	43	-50%
	5b3a17b	83	189	176%	326	384%	473	516%	161	90%	34	-54%
	Adione	4	7	83%	9	150%	12	232%	5	43%	3	-10%
HM	Andro	2800	5350	162%	9070	314%	12500	491%	5360	122%	3130	26%
	Etio	2370	4810	144%	7770	275%	10100	413%	5040	127%	2640	19%
	5en3b17a	89	90	2%	95	0%	58	-26%	54	-38%	75	-13%
	5a3a17a	11	10	9%	16	69%	10	35%	10	5%	9	2%
	5b3a17a	17	20	41%	30	105%	26	131%	23	56%	20	11%
	Epit	16	32	116%	58	284%	90	442%	28	73%	12	-28%
	T	47	190	328%	423	861%	680	1436%	146	205%	27	-46%
	5a3a17b	123	203	91%	423	277%	611	458%	306	180%	117	-10%
	5b3a17b	247	466	109%	1006	337%	1458	564%	676	182%	270	2%
	Adione	6	7	92%	12	194%	11	248%	6	39%	6	37%

Table 2. Mean urinary steroid excretion rates expressed as micrograms per day and percent change from baseline by day and group.

T excretion rate: HM vs. LM

		HM			
		Day 1	Day 2	Day 3	Day 4
LM	Day 1	0.6598			
	Day 2		0.0863		
	Day 3			0.0004	
	Day 4				<0.0001

Table 3: Statistical significance for between group comparisons for days 1 through 4 under the model T excretion rate = mode*day. P = 0.0002 for the overall model with $P < 0.05$ being significant.

TE: Hours required to exceed 4 and 6 and return to normal

	Subject	Infusion Interval* TE > 4	Infusion Interval* TE > 6	Post-infusion Interval** TE > 4	Post-infusion Interval** TE > 6
LM	1	Never	Never	Always	Always
	10	Never	Never	Always	Always
	12	58 -59	58 -59	0 - 4	0 - 4
	14	Never	Never	Always	Always
	15	Never	Never	Always	Always
	21	Never	Never	Always	Always
HM	3	32 -34	Never	0 - 7	Always
	4	0 - 3	27 -32	4 - 8	0 - 4
	5	0 - 4	4 - 8	13 -21	6 -13
	7	Always	4 - 8	4 - 8	0 - 4
	16	Always	Always	Never	9 -12
	17	0 - 3	29 -33	5 - 9	5 - 9

Table 4. Collection intervals (hours) from the beginning of infusion at which TE exceeds 4 and 6 and collection intervals (hours) from the end of the infusion until the T/E falls below 4 and 6 for individual subjects by group. Instances of "Never" and "Always" for the infusion and post-infusion intervals, respectively, indicate that a subject's TE did not exceed a given threshold during the infusion.

$\delta^{13}C$ Andro and Etio: Hours required to exceed population 3SD and return to normal

	Subject	Infusion Interval* Etio	Infusion Interval* Andro	Post-infusion Interval** Etio	Post-infusion Interval** Andro
LM	1	15 -23	4 - 8	4 - 8	4 - 8
	10	1 - 3	1 - 3	19 -21	8 -11
	12	8 -12	8 -12	8 -12	8 -12
	14	6 - 8	8 -16	11 -15	11 -15
	15	31 -35	31 -35	4 - 8	4 - 8
	21	26 -27	27 -32	4 - 6	4 - 6
HM	3	33 -34	33 -34	13 -15	7 -13
	4	24 -27	27 -32	8 -12	4 - 8
	5	4 - 8	4 - 8	21 -26	13 -21
	7	52 -56	32 -36	0 - 4	0 - 4
	16	14 -17	9 -14	7 - 9	4 - 7
	17	10 -13	10 -13	5 - 9	5 - 9

Table 5. Collection intervals (hours) from the beginning of infusion at which etiocholanolone and androsterone $\delta^{13}C$ values fall below their respective thresholds and the collection intervals (hours) from the end of the infusion until $\delta^{13}C$ values rise above the same thresholds for individual subjects by group.

$\delta^{13}C$ Androstanediols: Hours required to exceed population 3SD and return to normal

		Hours to decrease by 3SD				Hours to normal			
	Subject	5b-diol	5a-diol	5b-diff	5a-diff	5b-diol	5a-diol	5b-diff	5a-diff
LM	1	23 -28	8 -12	4 - 8	0 - 4	4 - 8	17 -25	8 -12	29 -32
	10	1 - 3	1 - 3	1 - 3	1 - 3	19 -20	20 -21	19 -20	19 -20
	12	6 - 8	6 - 8	6 - 8	6 - 8	12 -21	25 -29	12 -21	21 -25
	14	4 - 6	4 - 6	0 - 4	4 - 6	11 -15	15 -21	15 -21	15 -21
	15	9 -10	12 -13	6 - 9	12 -13	13 -28	13 -28	13 -28	13 -28
	21	38 -43	35 -38	17 -24	17 -24	0 - 4	0 - 4	0 - 4	0 - 4
HM	3	20 -23	18 -20	8 -11	8 -11	23 -33	33 -38	38 -41	38 -41
	4	35 -48	13 -24	7 -12	7 -12	12 -16	12 -16	31 -34	31 -34
	5	28 -32	4 - 8	4 - 8	4 - 8	13 -21	32 -36	32 -36	Never
	7	60 -74	32 -36	28 -32	32 -36	0 - 4	20 -24	24 -28	Never
	16	9 -14	0 - 9	14 -17	14 -17	26 -29	22 -26	26 -29	22 -26
	17	33 -37	10 -13	29 -33	10 -13	5 - 9	9 -12	9 -12	9 -12

Table 6. Collection intervals (hours) from the beginning of infusion at which the 5β and 5α-diol $\delta^{13}C$ values and their respective differences relative to 5β-Pdiol (ERC) fall outside their respective thresholds and the collection intervals (hours) from the end of the infusion until these markers return within the same thresholds for individual subjects by group. For HM subjects 5 and 7, the difference between the 5α-diol and ERC remained outside the threshold in the final collection interval.

TAB 36

REPORT to USADA on research subject IVT-1

April 16, 2007

Don H. Catlin, M.D.
President, Anti-Doping Research
Former Director, UCLA Olympic Analytical Laboratory

BACKGROUND

Over 98 percent of the carbon atoms in nature are ^{12}C but about 1.1% are ^{13}C, an isotope of carbon. If molecules of testosterone (T) or any other steroid are completely burned in a furnace at 800°C, the carbon atoms in the molecules are combusted to carbon dioxide. The gas chromatography-combustion-isotope ratio mass spectrometer (GC-C-IRMS) instrument measures the $^{13}C/^{12}C$ ratio of the combusted steroid, relative to a standard. The steroids are first extracted from urine, then separated in the GC, then combusted in the furnace, and finally the $\delta^{13}C$ value is determined.

There is a measurable difference in $\delta^{13}C$ values between pharmaceutical testosterone (T) manufactured from soy and other plant compounds and T biosynthesized by the human body. The $\delta^{13}C$ values of the former are approximately -30‰ and the values of the latter are approximately -25‰. This arises from differential 'fixing' of $^{13}CO_2$ and $^{12}CO_2$ in soy and related plants. (Krueger HW & Reesman RH 1982) Thus by determining the $\delta^{13}C$ value of urinary T or one or more of its metabolites one can determine its origin. (Aguilera et al. 1999)

In past years we have developed and optimized the IRMS method, (Aguilera et al. 1996; Aguilera et al. 1999; Aguilera et al. 2000; Aguilera et al. 2001) determined $\delta^{13}C$ values of various urinary steroids, validated our screening method which determines $\delta^{13}C$ values on androsterone and etiocholanolone, (Aguilera et al. 2000) and validated a confirmation method which determines the $\delta^{13}C$ values of two urinary diol metabolites of T, as well as of one endogenous reference steroid compound. (Aguilera et al. 1996; Aguilera et al. 2001) We have also presented evidence that the IRMS method is useful for distinguishing cases of naturally elevated testosterone to epitestosterone ratio (T/E) (Aguilera et al. 2001) from T users. Others have confirmed (Shackleton et al. 1997a) and extended our studies. (Shackleton et al. 1997b; Ueki & Okano 1999) Our results show, as expected, that T administration lowers $\delta^{13}C$ values of urine T metabolites. All the previous studies delve into this method, its efficacy, and the $\delta^{13}C$ values of healthy subjects, whereas this study probes the underlying physiology, endocrinology, and pharmacology of the application of the IRMS method.

SPECIFIC AIM

To conduct a pharmacokinetic study of T using $\delta^{13}C$ values, T/E ratios, T metabolite concentrations, and other variables on urine samples collected from a healthy male volunteer who received a zero-order infusion (constant dose) of testosterone for three days preceded by a one-day baseline period.

METHOD

The subject (IVT-1) was a healthy 22 year old male who volunteered to participate in a UCLA Clinical Research Center study designed to characterize the effects of intravenous T on the hypothalamic-pituitary-testicular axis and the excretion of T and its metabolites. The study was approved by the UCLA Institutional Review Board and this subject gave written informed consent. IVT-1 completed a medical screening questionnaire: he was free from any medical problems and had no illnesses affecting his kidneys, liver or reproductive hormones. He was not taking any prescription medications or nutritional supplements.

Prior to the infusion, his urinary baseline T/E was determined from a single urine sample. It was less than 0.3. After collection of a baseline urine (study day 1), the subject received a continuous intravenous infusion of T. On the first day of the T infusion (study day 2) the dose was 7 $mg/m^2/24h$, which is the approximate T production rate of an average male. On the second day of the T infusion (study day 3), the dose was doubled to 14 $mg/m^2/24h$. On the third day of the T infusion (day 4), the dose was doubled again to 28 $mg/m^2/24h$. On days 5 and 6, the post infusion decline in T/E and $\delta^{13}C$ values were determined. All urine was collected on each experimental day in timed intervals ranging nominally from 3 to 8 hours.

The details of the analytical methods have been published. (Aguilera et al. 1996; Aguilera et al. 1999; Aguilera et al. 2000; Aguilera et al. 2001) The urinary concentration of T was determined by GC/MS and is expressed in ng/mL. The and $\delta^{13}C$ values are expressed in ‰ and were determined by GC-C-IRMS. The $\delta^{13}C$ values were determined for five urinary steroids: androsterone, etiocholanolone, 5β-androstane-3α,17β-diol (abbreviated, 5b-Adiol) diacetate, 5α-androstane-3α,17β-diol (5a-Adiol) diacetate, and 5β-pregnane-3α,20α-diol (Pdiol) diacetate.

RESULTS

The testosterone used for the infusion was found to have a $\delta^{13}C$ value of -29.3 ± 0.1‰. Epitestosterone was not detected in the material.

Table 1 summarizes the dose administered and the urine steroid concentrations by hour for subject IVT-1. The dose is determined from the subject's body surface area (BSA). Note that on Day 2 when the T infusion takes place the concentrations of urinary steroids increase, and remain elevated until the infusion is discontinued on day 5. It was expected that the T would increase.

Table 1. Concentrations of six urinary steroids for subject IVT-1 during the six day infusion study

			CONCENTRATIONS (ng/mL)					
DAY	mg per BSA	HOURS	ANDRO	ETIO	T	E	5a-Adiol	5b-Adiol
1	0	0 - 4	3780	3450	3	25	116	111
		4 - 8	2380	2230	2	14	85	64
		8 - 12	2400	2420	2	16	82	72
		12 - 15	1660	2020	2	11	59	55
		15 - 19	3290	3740	3	22	117	96
		19 - 23	2890	2910	3	21	102	91
2	7	23 - 27	1430	1270	2	9	35	29
		27 - 31	1240	980	3	11	48	34
		31 - 35	4980	3410	9	31	172	124
		35 - 38	5420	3920	11	31	211	152
		38 - 46	9970	7310	25	66	452	300
3	14	46 - 51	6770	5300	16	51	302	247
		51 - 55	2540	1680	5	15	69	52
		55 - 58	4170	2990	10	30	149	105
		58 - 62	3710	2550	9	24	118	76
		62 - 70	12700	9270	39	105	674	466
4	28	70 - 75	6400	4680	16	45	243	168
		75 - 79	5510	3670	16	41	193	128
		79 - 83	7080	4490	16	49	217	135
		83 - 86	2850	1970	8	27	98	63
		86 - 94	14200	9410	41	144	687	423
5	0	94 - 98	15000	11600	32	144	1020	676
		98 - 102	2780	3020	2	14	154	87
		102 - 106	2710	3760	1	10	120	85
		106 - 111	1190	1720	1	4	31	25
		111 - 119	2090	2930	2	14	63	56
6	0	119 - 123	1400	1680	1	6	25	27
		123 - 126	1050	980	1	5	18	16
		126 - 131	1140	940	1	5	18	13
		131 - 135	780	930	1	5	15	15
		135 - 142	2200	2680	2	21	62	57

Day = study day
mg per BSA = mg T infused per Body Surface Area (measured in meters squared)
Andro=androsterone
Etio=etiocholanolone
T = testosterone
E = epitestosterone

Table 2 summarizes the dose administered, T/E, and $\delta^{13}C$ values by hour for subject IVT-1. Note that this individual's T/E approximately doubles during the T infusion, but the absolute values remain at 0.4 or less. Also note that the $\delta^{13}C$ values of the T metabolites become more negative during the infusion, whereas the Pdiol values do not.

Table 2. Delta values of five urinary steroids for subject IVT-1 during the six-day T infusion study and differences in delta values between 5a-Adiol and Pdiol, and 5b-Adiol and Pdiol.

				$d^{13}C$ (per mil)					Difference in d13C values (per mil)	
DAY	mg per BSA	HOURS	T/E	ANDRO	ETIO	5a-Adiol DIACETATE	5b-Adiol DIACETATE	Pdiol DIACETATE	5a-Adiol diacetate - Pdiol diacetate	5b-Adiol diacetate - Pdiol diacetate
1	0	0 - 4	0.14	-19.5	-21.2	-25.0	-22.8	-23.1	-1.9	0.3
		4 - 8	0.15	-18.9	-20.6	-25.3	-23.6	-23.3	-2.0	-0.3
		8 - 12	0.13	-19.6	-20.5	-25.7	-23.8	-23.3	-2.4	-0.5
		12 - 15	0.16	-19.6	-20.5	-25.7	-23.5	-23.1	-2.6	-0.4
		15 - 19	0.15	-19.0	-20.6	-24.2	-23.0	-22.5	-1.7	-0.5
		19 - 23	0.15	-19.2	-20.9	-24.8	-23.2	-22.9	-1.9	-0.3
2	7	23 - 27	0.27	-22.3	-23.1	-26.6	-25.5	-22.6	-4.0	-2.9
		27 - 31	0.33	-24.0	-24.8	-27.9	-27.0	-22.7	-5.2	-4.3
		31 - 35	0.32	-24.6	-25.4	-29.3	-28.3	-23.0	-6.3	-5.3
		35 - 38	0.37	-25.3	-24.9	-29.2	-27.3	-22.9	-6.3	-4.4
		38 - 46	0.40	-25.5	-26.4	-30.0	-28.2	-22.8	-7.2	-5.4
3	14	46 - 51	0.33	-24.6	-25.4	-29.5	-29.0	-22.7	-6.8	-6.3
		51 - 55	0.38	-25.5	-26.9	-30.2	-28.0	-22.9	-7.3	-5.1
		55 - 58	0.37	-26.2	-26.9	-29.4	-28.5	-22.6	-6.8	-5.9
		58 - 62	0.41	-27.1	-27.4	-30.1	-28.3	-22.3	-7.8	-6.0
		62 - 70	0.38	-26.2	-27.3	-29.9	-29.1	-22.3	-7.6	-6.8
4	28	70 - 75	0.38	-26.3	-27.5	-30.1	-29.5	-22.4	-7.7	-7.1
		75 - 79	0.40	-26.7	-28.1	-31.0	-29.1	-22.6	-8.4	-6.5
		79 - 83	0.34	-26.6	-28.1	-30.3	-29.4	-22.5	-7.8	-6.9
		83 - 86	0.33	-27.2	-28.3	-30.0	-30.5	-22.7	-7.3	-7.8
		86 - 94	0.30	-27.0	-28.3	-30.2	-29.9	-22.6	-7.6	-7.3

		94 - 98	0.23	-26.1	-26.7	-30.2	-29.0	-22.9	-7.3	-6.1
		98 - 102	0.16	-23.7	-24.1	-30.4	-27.0	-22.2	-8.2	-4.8
5	0	102 - 106	0.11	-22.2	-22.8	-29.6	-25.6	-22.7	-6.9	-2.9
		106 - 111	0.13	-22.4	-22.8	-28.7	-24.8	-22.6	-6.1	-2.2
		111 - 119	0.14	-21.1	-21.9	-27.2	-24.9	-22.7	-4.5	-2.2
		119 - 123	0.17	-20.5	-21.8	-26.7	-23.8	-22.5	-4.2	-1.3
		123 - 126	0.14	-20.5	-22.0	-26.4	-24.0	-22.6	-3.8	-1.4
6	0	126 - 131	0.21	-20.8	-22.2	-27.0	-24.0	-22.9	-4.1	-1.1
		131 - 135	0.20	-20.9	-21.8	-27.1	-25.0	-22.8	-4.3	-2.2
		135 - 142	0.11	-20.5	-21.8	-25.9	-23.8	-22.5	-3.4	-1.3

Notes:

Day = study day

mg per BSA = mg T infused per body surface area measured in meters squared

Andro = androsterone, underivatized

Etio = etiochlanolone underivatized

T/E = testosterone / epitestosterone

Acetylation reagent $d^{13}C$ value: -37.35

Figure 1a and 1b depict the pharmacodynamic response of the T/E and the $\delta^{13}C$ values for androsterone and etiocholanolone before, during, and following the three day T infusion. As expected the T/E values begin to increase after the T infusion begins. Likewise the $\delta^{13}C$ values for the diol metabolites diacetates (not shown as a graph), androsterone, and etiocholanolone decrease as the infusion of T commences and return to baseline at the end of the infusion.

Figure 1c plots the differences in $\delta^{13}C$ values between the 5a-Adiol and Pdiol, and between the 5b-Adiol and Pdiol. Note that the 5aAdiol - Pdiol differences in the first 24 hours are between -1.8 and -2.6, decrease on days 2, 3, and 4, and then increase as they return to baseline. Note that compared to the 5b-Adiol, the 5a-Adiol differences are slower to return to baseline in the first day following the end of the infusion. As a consequence the gaps between the 5b-Adiol and 5a-Adiol differences are the most pronounced in the first 24 hours after the infusion is discontinued.

Fig 1a

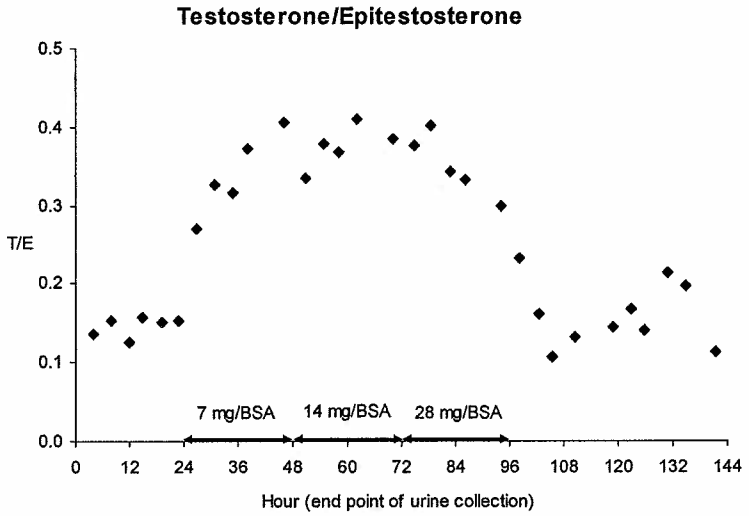


Fig 1b

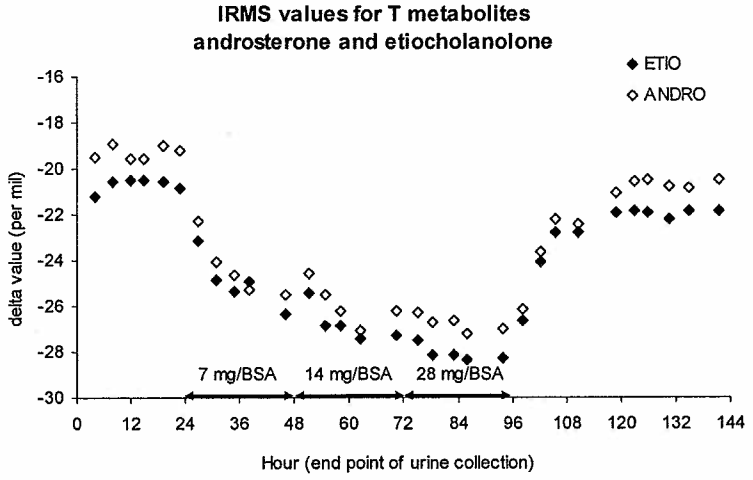
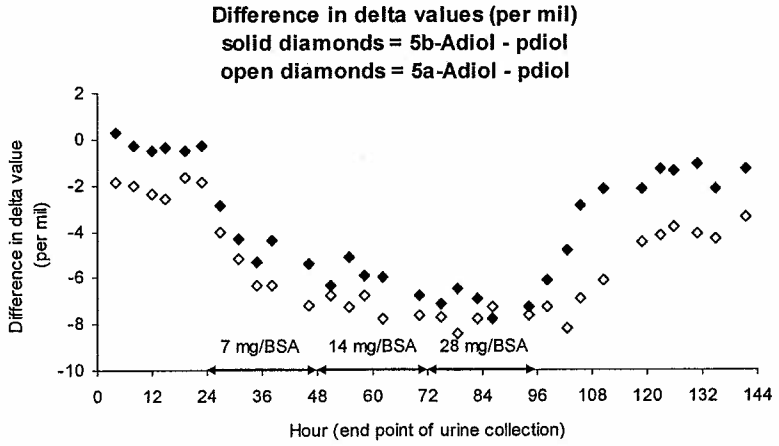


Fig 1c



REFERENCES

Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B & Pope HG, Jr. 1996 Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. J Mass Spectrom. 1996;31:169-176.

Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, Swerdloff RS, Pope HG & Hatton CK. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. J.Chromatogr B Biomed Sci Appl. 1999;727: 95-105.

Aguilera R, Chapman TE & Catlin DH 2000 A rapid screening assay for measuring urinary androsterone and etiocholanolone delta(13)C (per thousand) values by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom.2000;14(23)2294-9.

Aguilera R, Chapman TE, Starcevic B, Hatton CK & Catlin DH 2001 Performance characteristics of a carbon isotope ratio method for detecting doping with testosterone based on urine diols: controls and athletes with elevated testosterone/epitestosterone ratios. Clin Chem 2001;47(2):292-300.

Krueger HW and Reesman RH. Carbon Isotope analyses in food technology. Mass Spectrometry Reviews 1982;1:205-236.

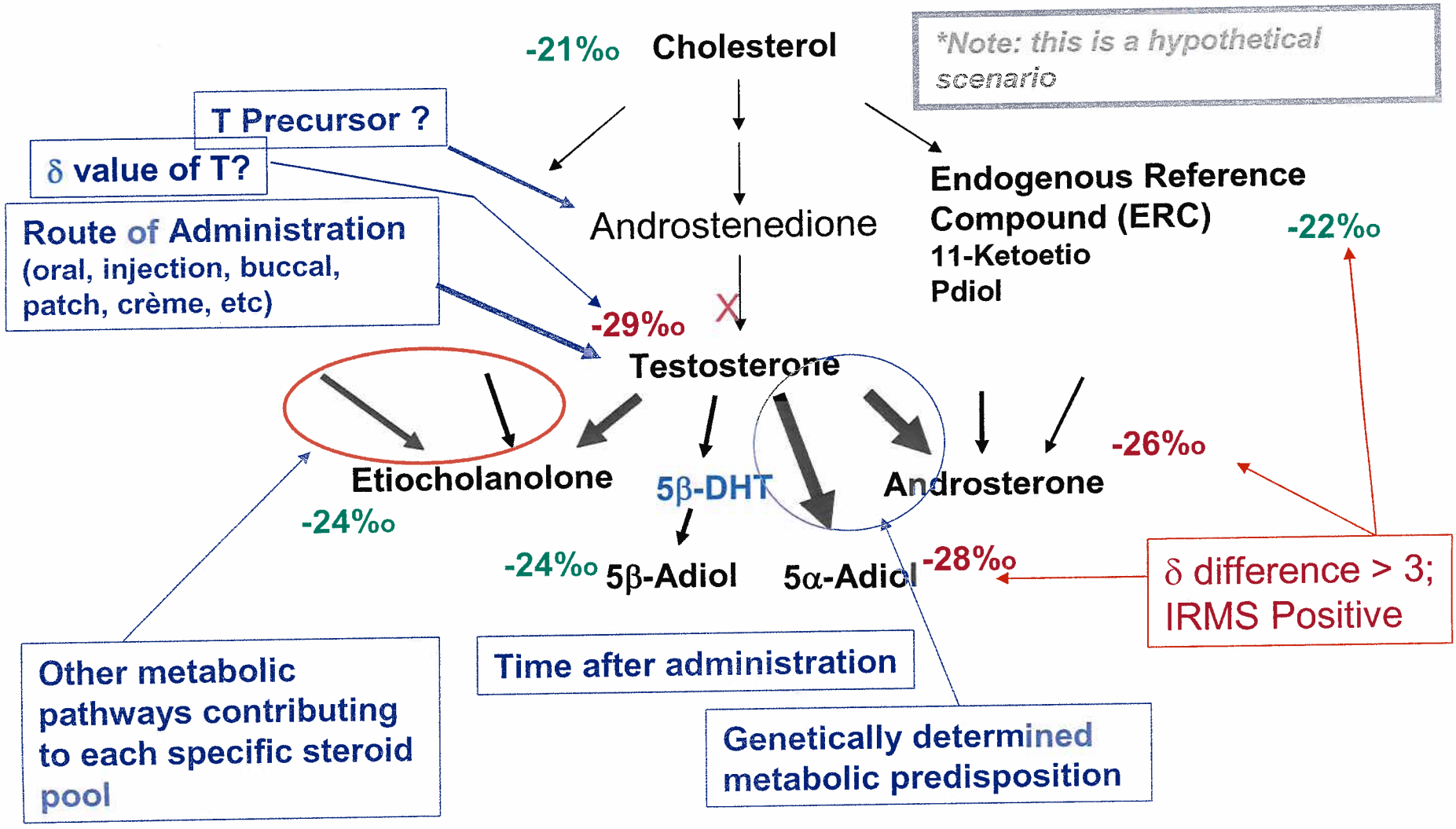
Shackleton CH, Phillips A, Chang T & Li Y. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids 1997a;62:379-387.

Shackleton CH, Roitman E, Phillips A & Chang T Androstanediol and 5-andro-stenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: potential use in gas chromatography isotope ratio mass spectrometry. Steroids 1997b; 62:665-673.

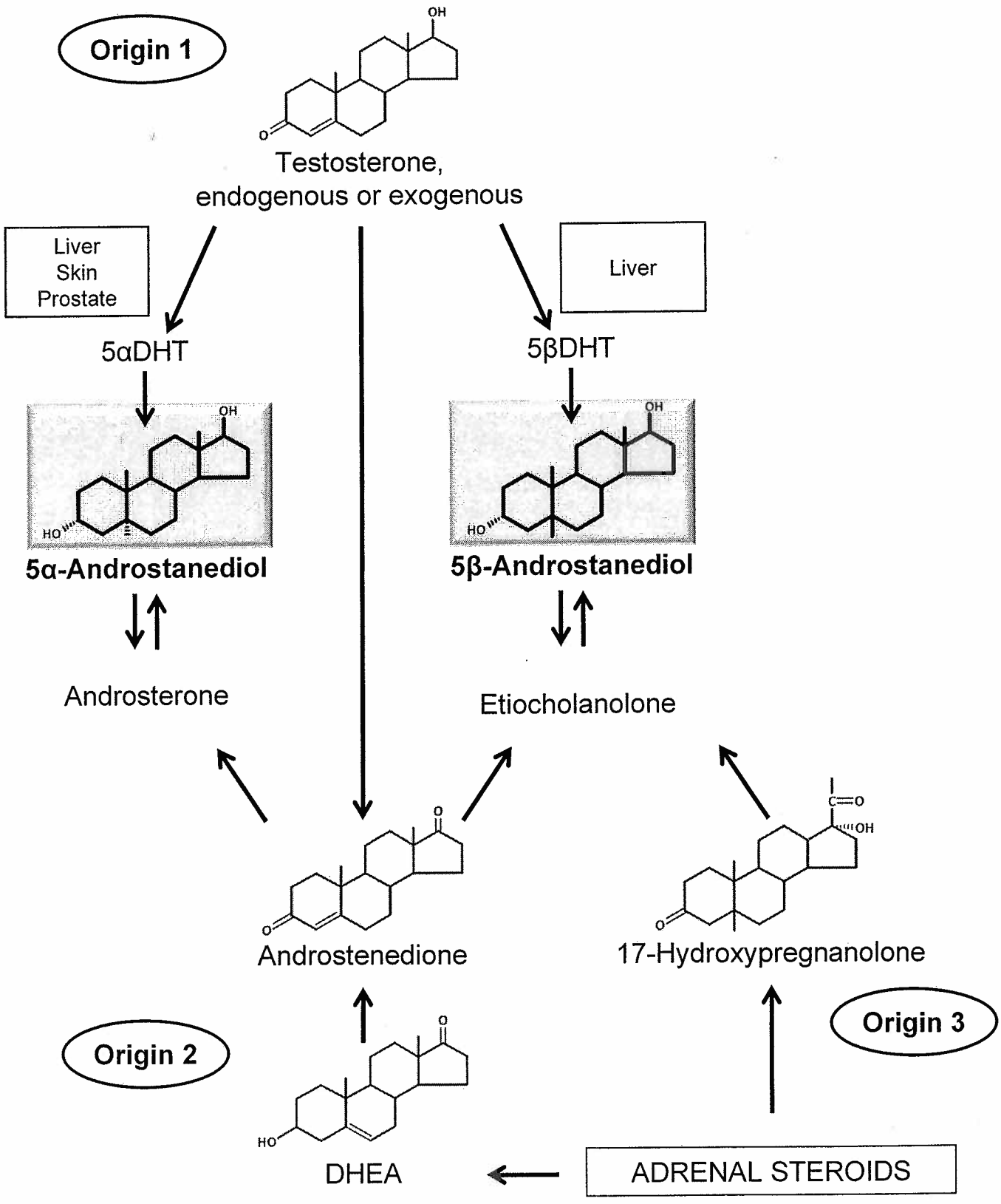
Ueki M & Okano M. Analysis of exogenous dehydroepiandrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun.Mass Spectrom 1999;13:2237-2243.

TAB 37

Figure 3.
Pharmacological and Biological Complexity in Isotope Ratio Mass Spec Test



The Multiple Origins of the Andostanediols, Androsterone and Etiocholanolone



This is a simplified chart and does not contain all known metabolic and synthetic reactions.

TAB 38

Figure 1.
Simplified Illustration of IRMS Test for Testosterone
(No Doping with Exogenous Testosterone)

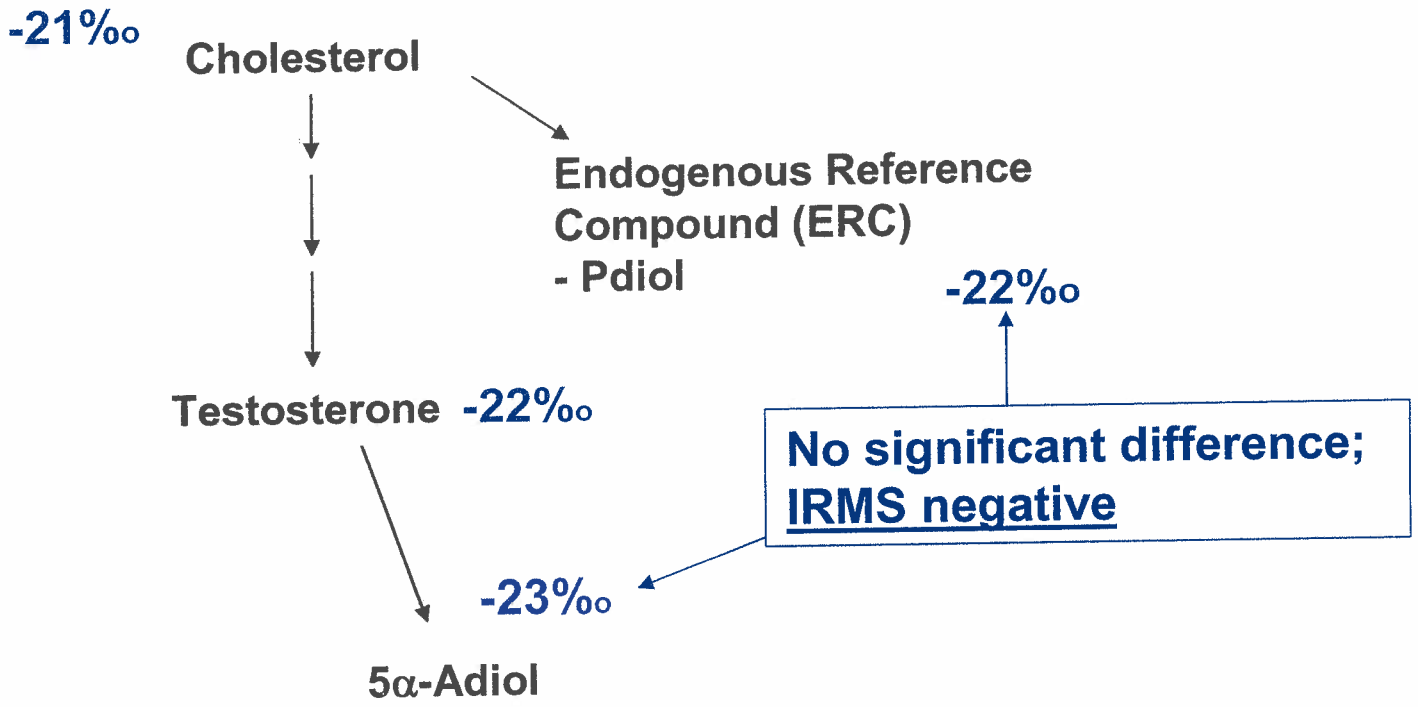


Figure 2.
Simplified Illustration of IRMS Test for Testosterone
(Doping with Exogenous Testosterone)

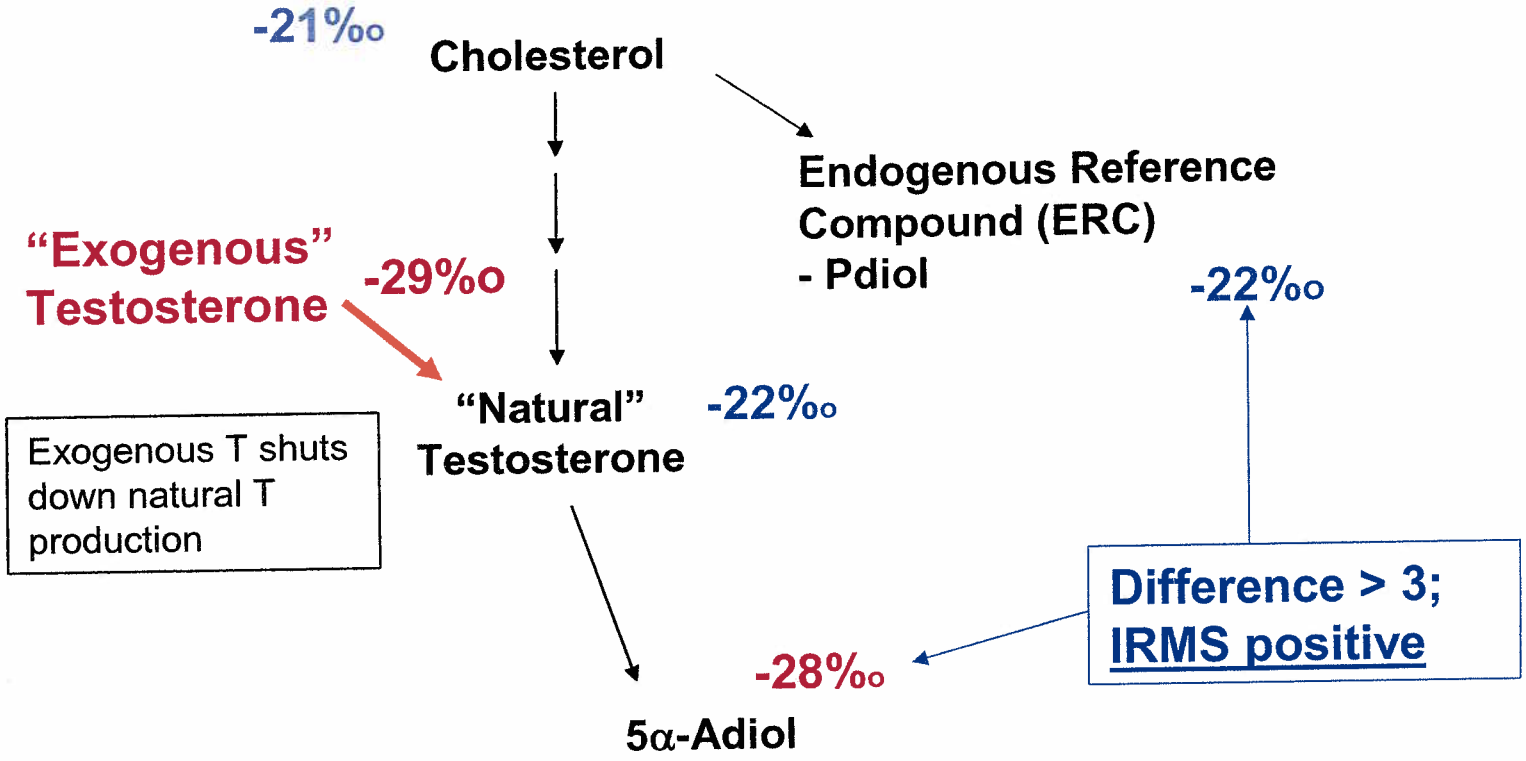


Figure 1

MIX CAL IRMS Delta values (per mil)					
A sample IRMS confirmation					
Data filename	decane	undecane	dodecane	methyldecanoate	Delta values: USADA page
DATA_004	-32.52	-28.45	-31.96	-31.51	0178
DATA_005	-32.74	-28.28	-32.13	-31.76	0179
DATA_006	-32.56	-28.65	-31.95	-31.59	0180
B sample IRMS confirmation					
Data filename	decane	undecane	dodecane	methyldecanoate	Delta values: USADA page
DATA_006	-32.30	-27.78	-31.79	-31.30	0357
DATA_007	-32.44	-27.99	-31.93	-31.44	0358
DATA_008	-32.33	-27.81	-31.80	-31.22	0359

Figure 2

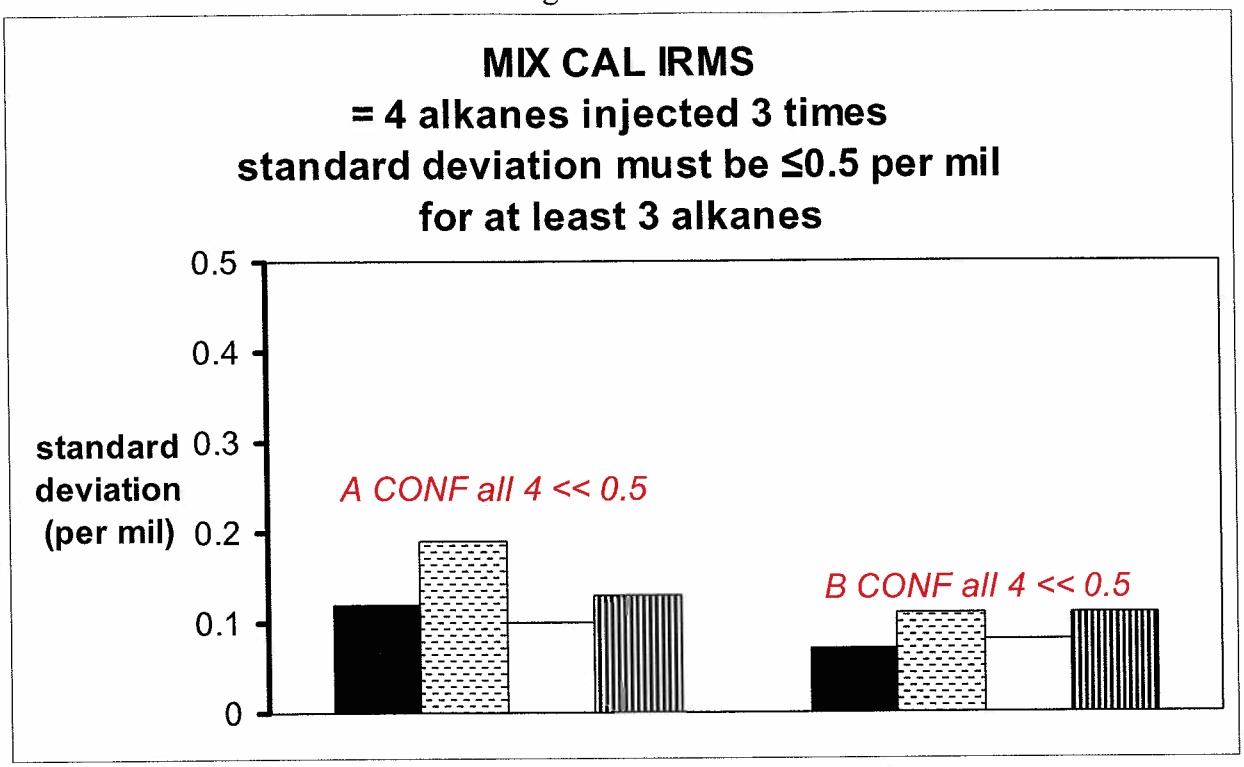

MIX CAL IRMS
= 4 alkanes injected 3 times
standard deviation must be ≤0.5 per mil
for at least 3 alkanes
standard deviation (per mil)
0.5
0.4
0.3
0.2
0.1
0
A CONF all 4 << 0.5
B CONF all 4 << 0.5

Figure 3. Accuracy of Mixed Cal Acetate δ Values

	5α-Androstanol	Etiocholanolone	5β-Androstanediol	11-Ketoetiocholanolone
"True" Value from Eurofins	-30.46 ‰	-19.91 ‰	-33.81 ‰	-16.30 ‰
7/24 immediately before Respondent's sample	-30.29 ‰	-20.01 ‰	-33.70 ‰	-16.69 %
7/24 immediately after Respondent's sample	-30.56 ‰	-20.22 ‰	-33.90 ‰	-16.76 ‰
8/4 immediately before Respondent's sample	-30.40 ‰	-19.98 ‰	-33.71 ‰	-16.74 ‰
8/4 immediately after Respondent's sample	-30.25 ‰	-19.95 ‰	-33.63 ‰	-16.68 ‰

Figure 4

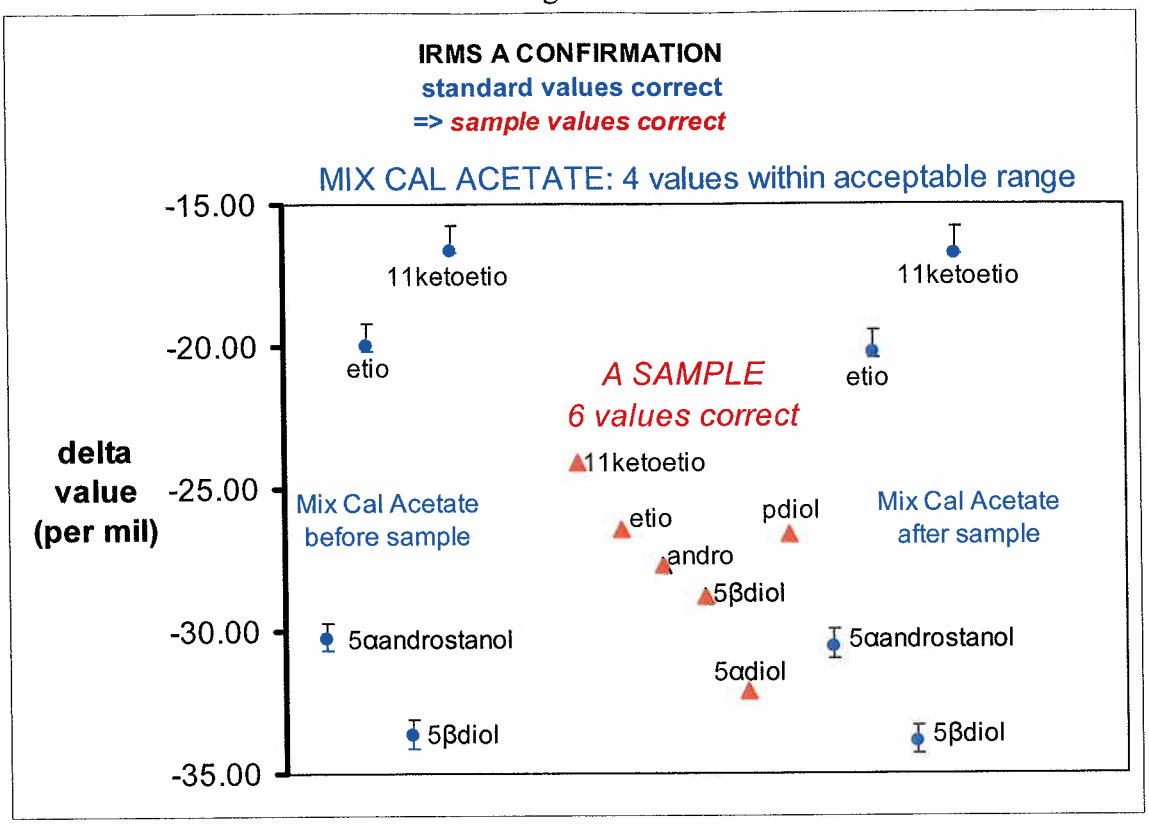

IRMS A CONFIRMATION
standard values correct
=> sample values correct
MIX CAL ACETATE: 4 values within acceptable range
delta value (per mil)
-15.00
-20.00
-25.00
-30.00
-35.00
11ketoetio
etio
Mix Cal Acetate before sample
5αandrostanol
5βdiol
A SAMPLE
6 values correct
11ketoetio
etio
pdiol
andro
5βdiol
5αdiol
11ketoetio
etio
Mix Cal Acetate after sample
5αandrostanol
5βdiol

Figure 5

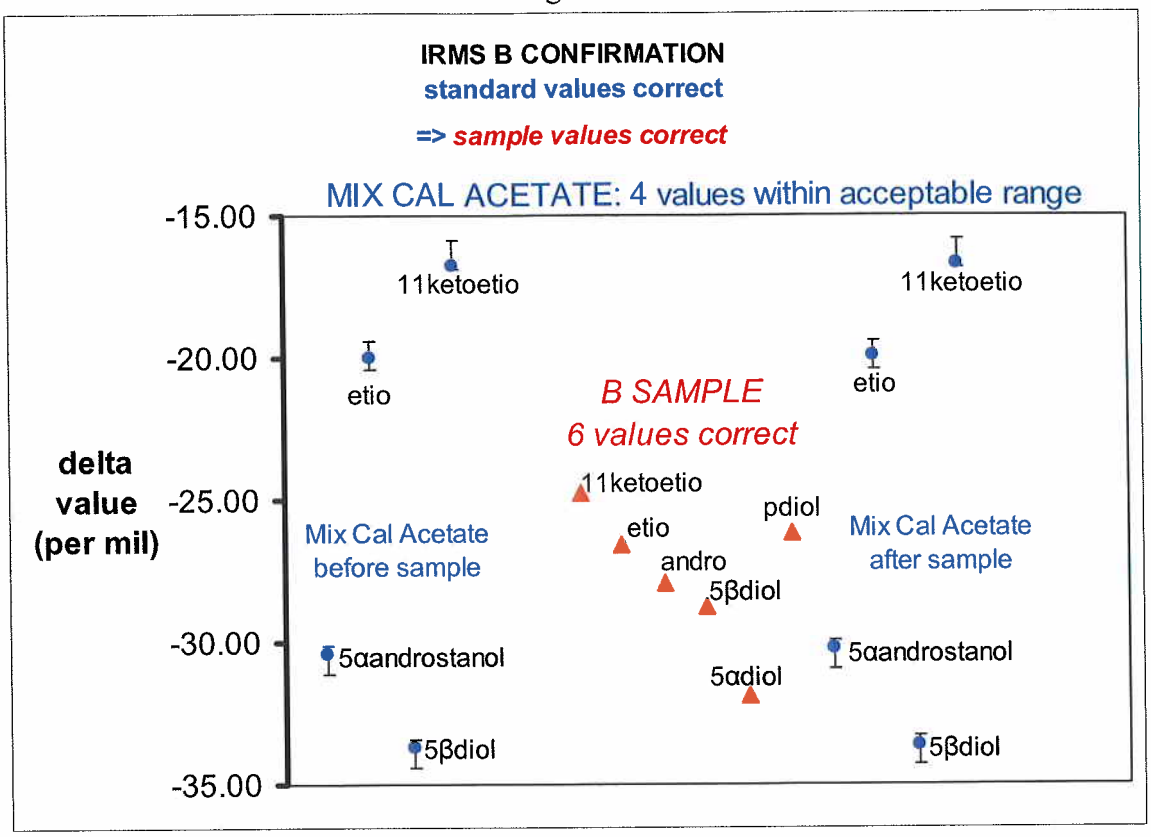

IRMS B CONFIRMATION
standard values correct
=> sample values correct
MIX CAL ACETATE: 4 values within acceptable range
delta value (per mil)
-15.00
-20.00
-25.00
-30.00
-35.00
11ketoetio
etio
5αandrostanol
5βdiol
Mix Cal Acetate before sample
B SAMPLE
6 values correct
11ketoetio
etio
pdiol
andro
5βdiol
5αdiol
Mix Cal Acetate after sample
11ketoetio
etio
5αandrostanol
5βdiol

Figure 6

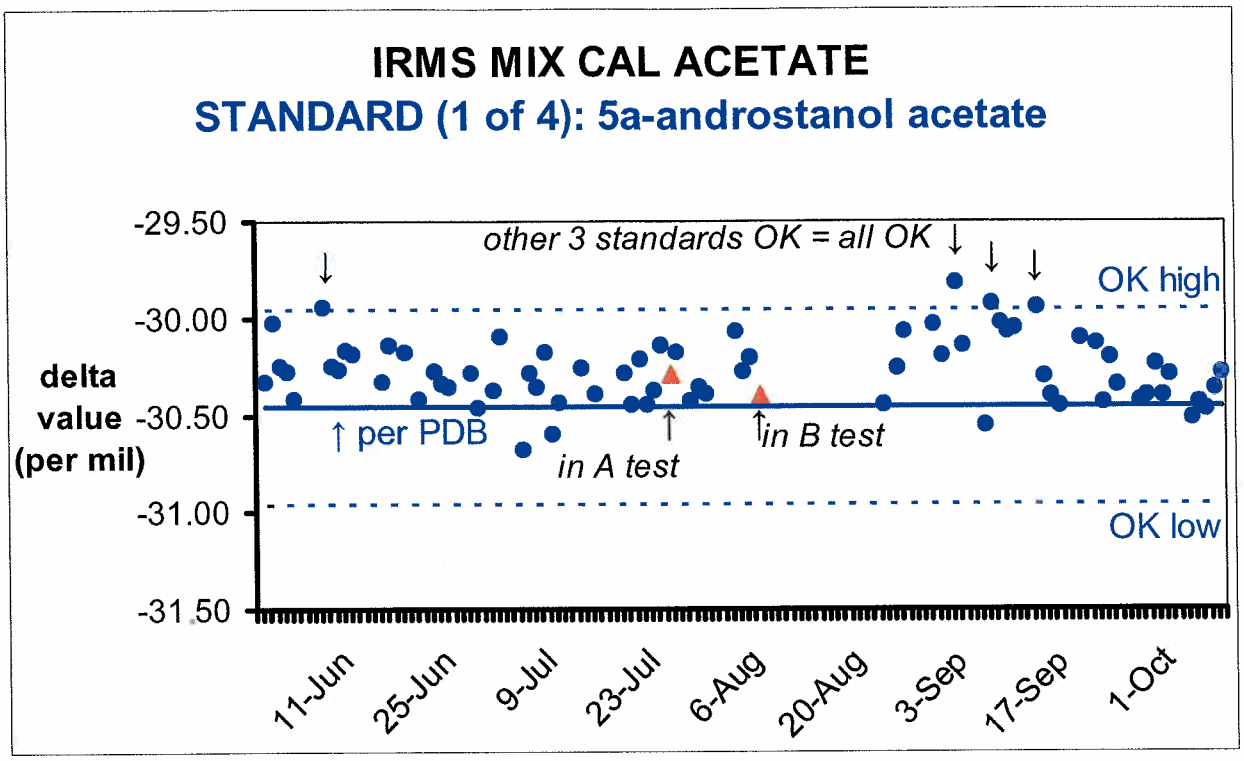

IRMS MIX CAL ACETATE
STANDARD (1 of 4): 5a-androstanol acetate
-29.50
-30.00
-30.50
-31.00
-31.50
delta value (per mil)
other 3 standards OK = all OK
OK high
OK low
↑ per PDB
in A test
in B test
11-Jun
25-Jun
9-Jul
23-Jul
6-Aug
20-Aug
3-Sep
17-Sep
1-Oct

Figure 7

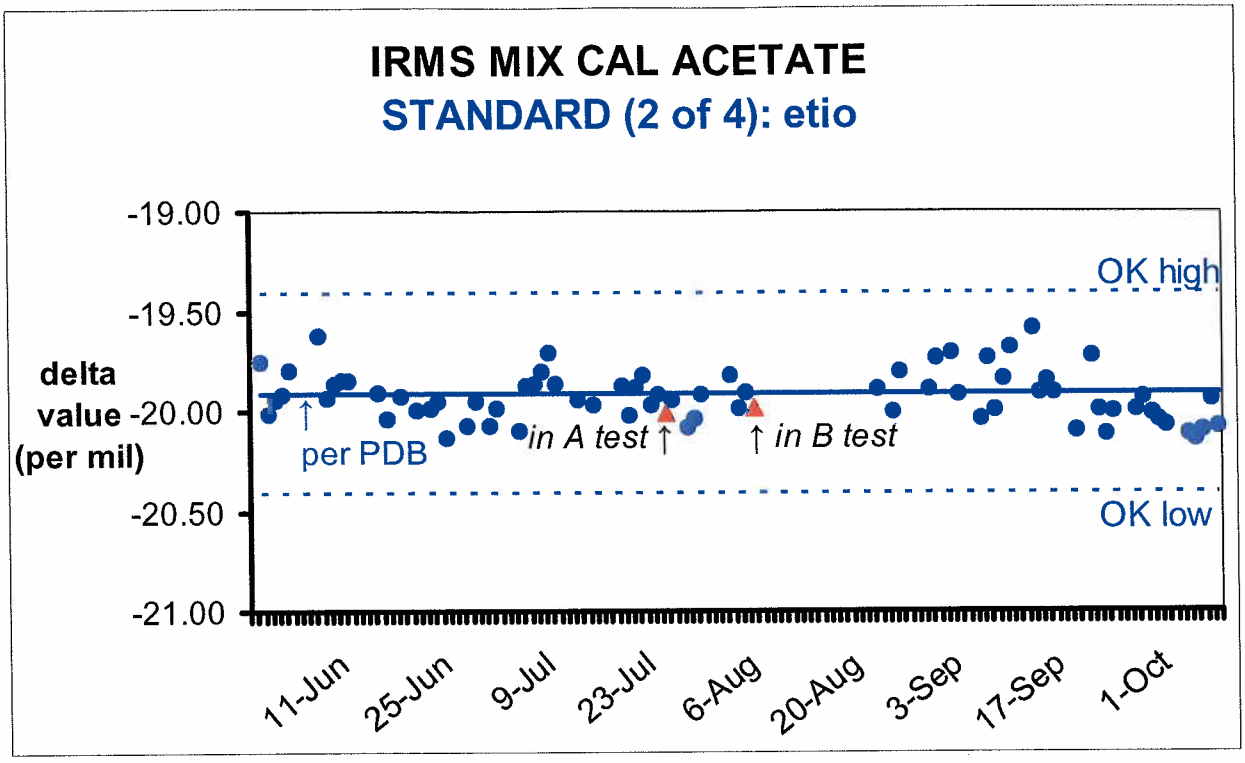

IRMS MIX CAL ACETATE
STANDARD (2 of 4): etio
-19.00
-19.50
-20.00
-20.50
-21.00
delta value (per mil)
OK high
OK low
per PDB
in A test
in B test
11-Jun
25-Jun
9-Jul
23-Jul
6-Aug
20-Aug
3-Sep
17-Sep
1-Oct

Figure 8

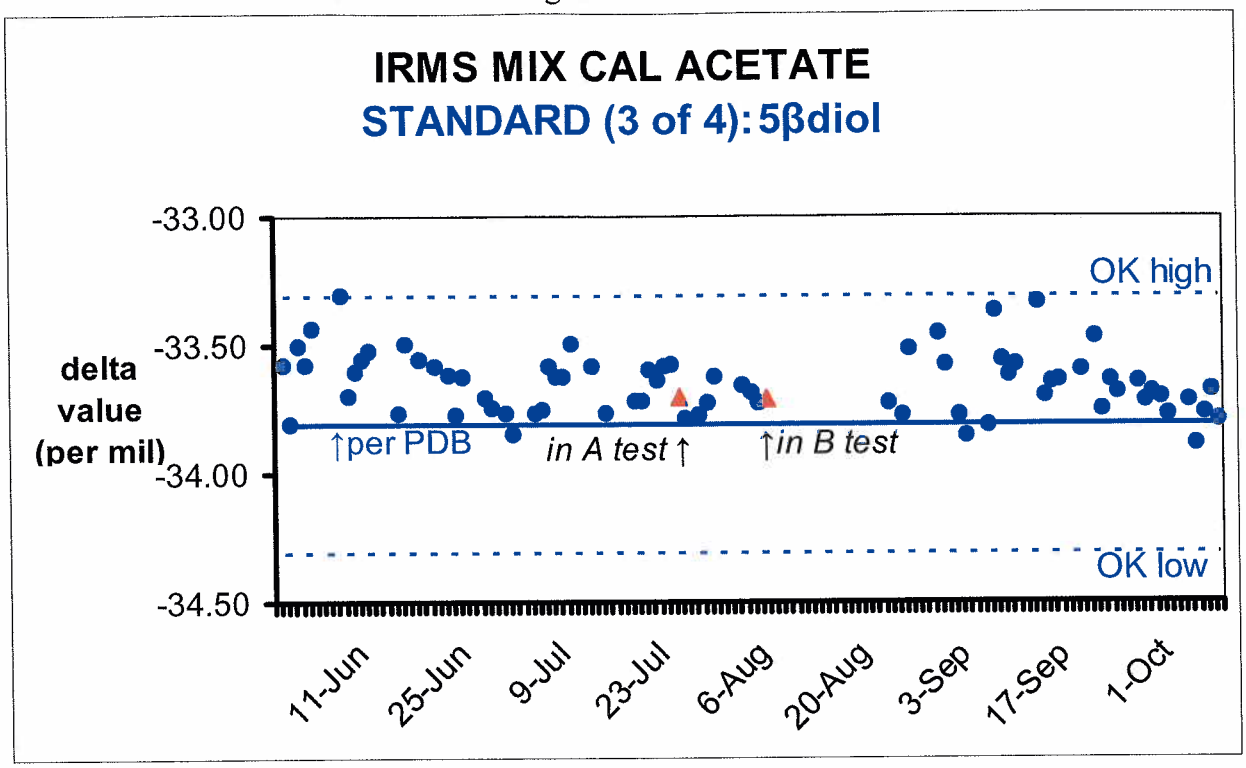

IRMS MIX CAL ACETATE
STANDARD (3 of 4): 5βdiol
delta value (per mil)
-33.00
-33.50
-34.00
-34.50
OK high
OK low
↑per PDB
in A test ↑
↑in B test
11-Jun
25-Jun
9-Jul
23-Jul
6-Aug
20-Aug
3-Sep
17-Sep
1-Oct

Figure 9

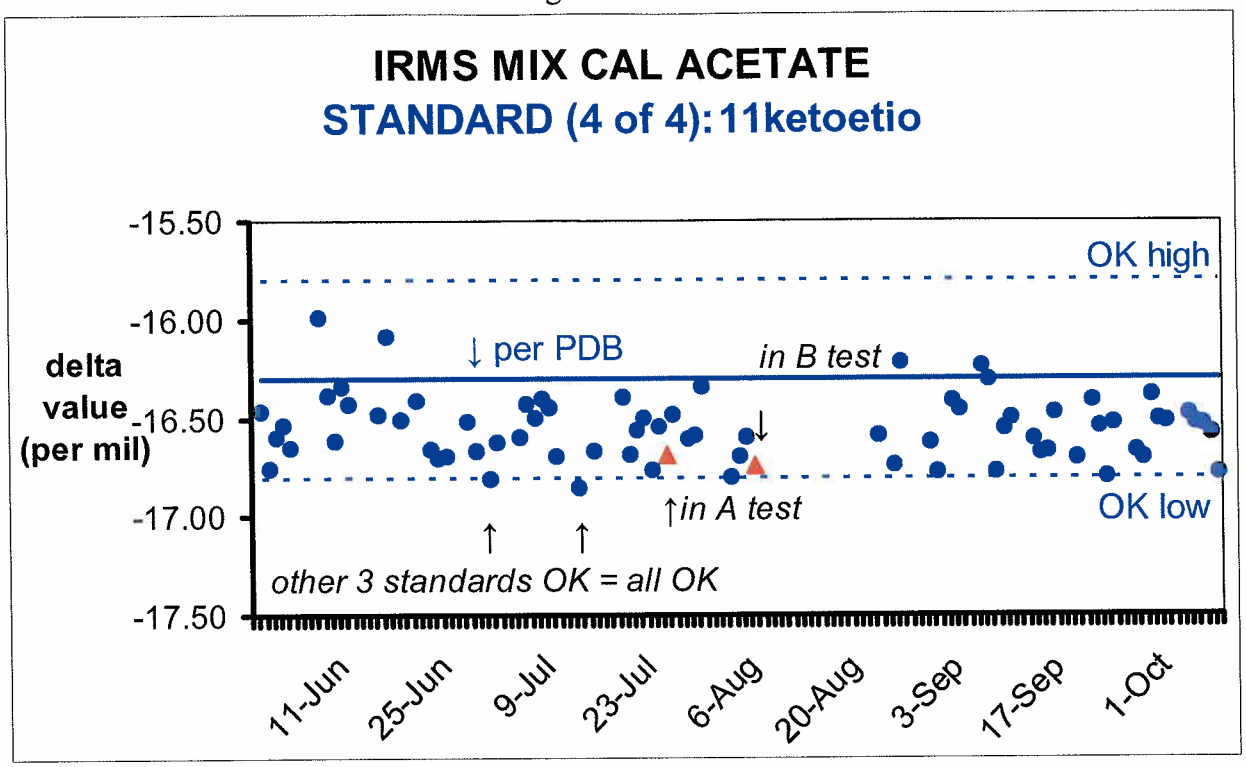

IRMS MIX CAL ACETATE
STANDARD (4 of 4): 11ketoetio
-15.50
-16.00
-16.50
-17.00
-17.50
delta value (per mil)
OK high
↓ per PDB
in B test
↓in A test
OK low
other 3 standards OK = all OK
11-Jun
25-Jun
9-Jul
23-Jul
6-Aug
20-Aug
3-Sep
17-Sep
1-Oct

Figure 10

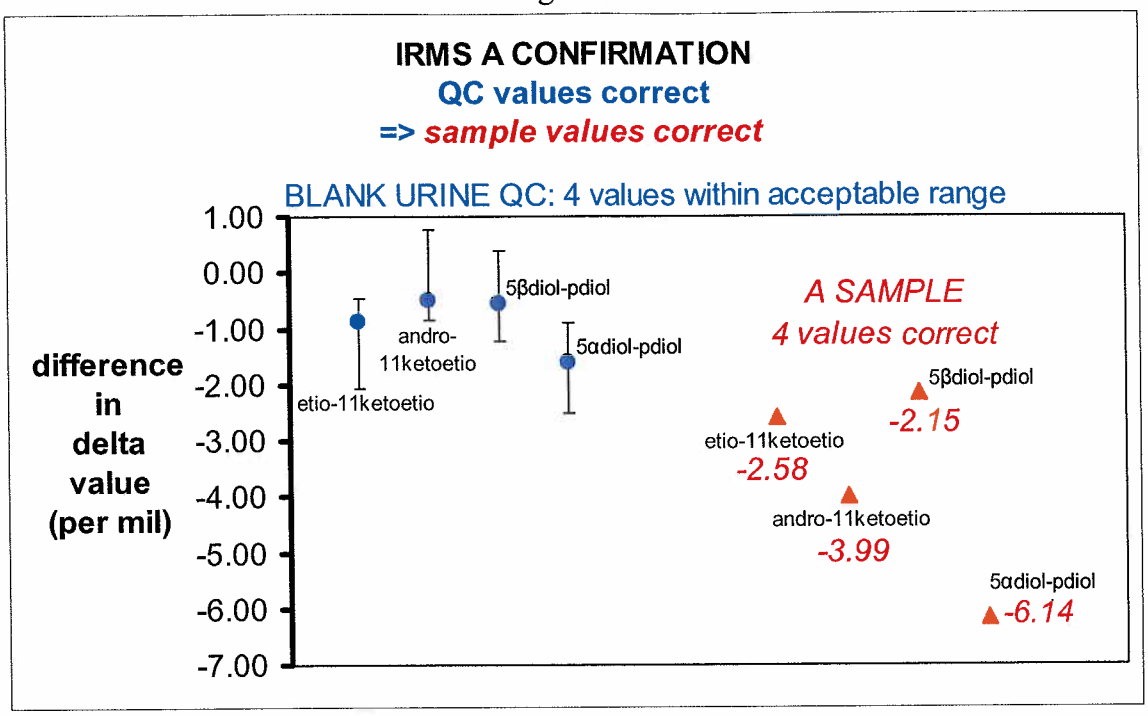

IRMS A CONFIRMATION
QC values correct
=> sample values correct
BLANK URINE QC: 4 values within acceptable range
difference in delta value (per mil)
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
-6.00
-7.00
etio-11ketoetio
andro-11ketoetio
5βdiol-pdiol
5αdiol-pdiol
A SAMPLE
4 values correct
5βdiol-pdiol
-2.15
etio-11ketoetio
-2.58
andro-11ketoetio
-3.99
5αdiol-pdiol
-6.14

Figure 11

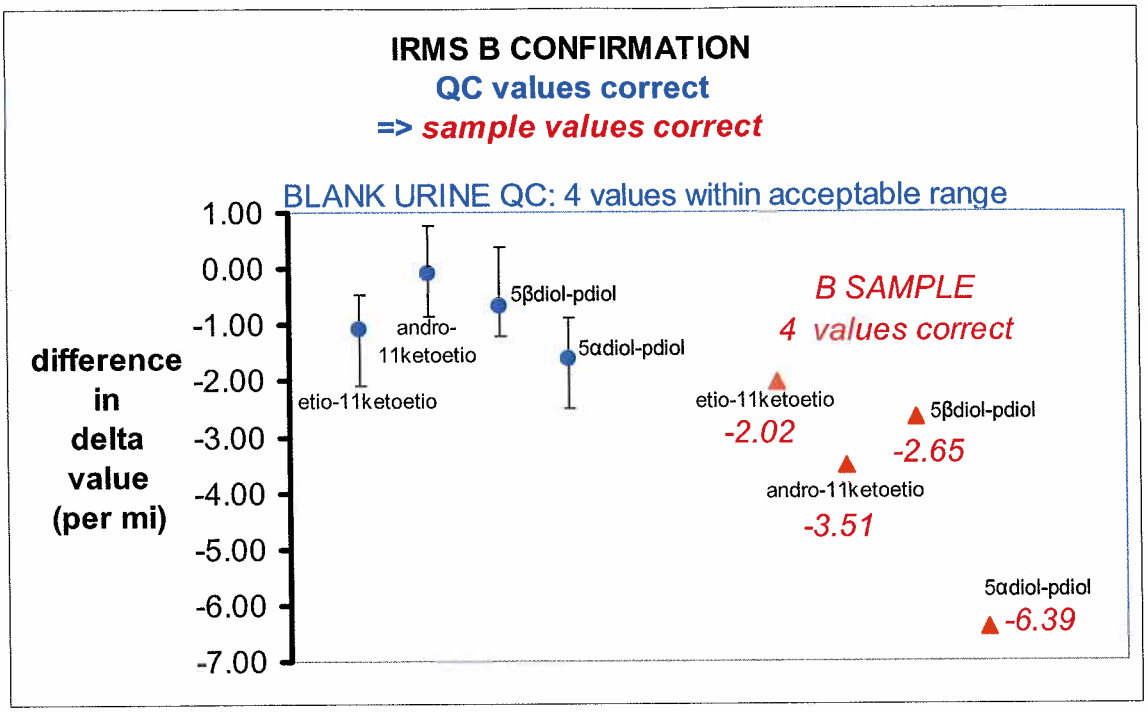

IRMS B CONFIRMATION
QC values correct
=> sample values correct
BLANK URINE QC: 4 values within acceptable range
difference in delta value (per mi)
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
-6.00
-7.00
etio-11ketoetio
andro-11ketoetio
5βdiol-pdiol
5αdiol-pdiol
B SAMPLE
4 values correct
etio-11ketoetio
-2.02
5βdiol-pdiol
-2.65
andro-11ketoetio
-3.51
5αdiol-pdiol
-6.39

Figure 12

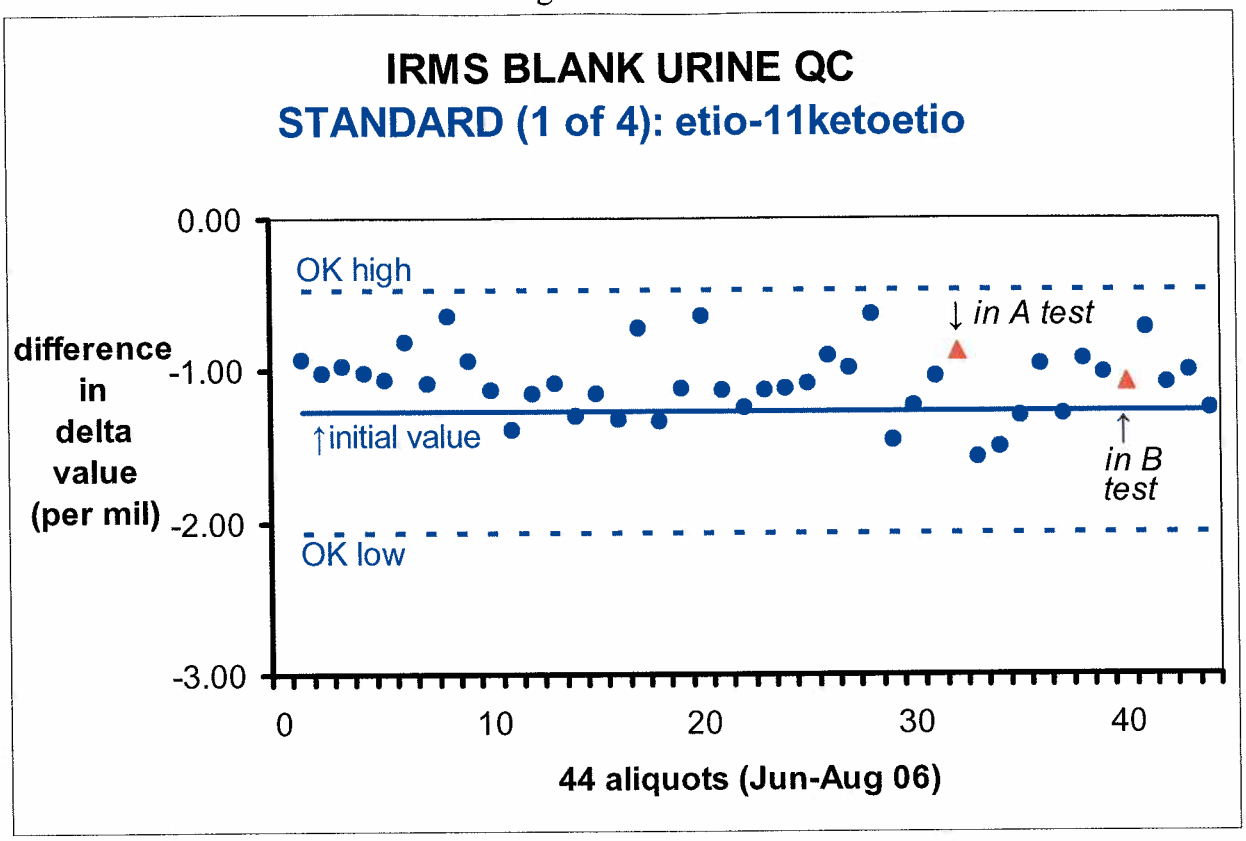

IRMS BLANK URINE QC
STANDARD (1 of 4): etio-11ketoetio
0.00
-1.00
-2.00
-3.00
difference in delta value (per mil)
OK high
OK low
↓ in A test
↑initial value
in B test
0
10
20
30
40
44 aliquots (Jun-Aug 06)

Figure 13

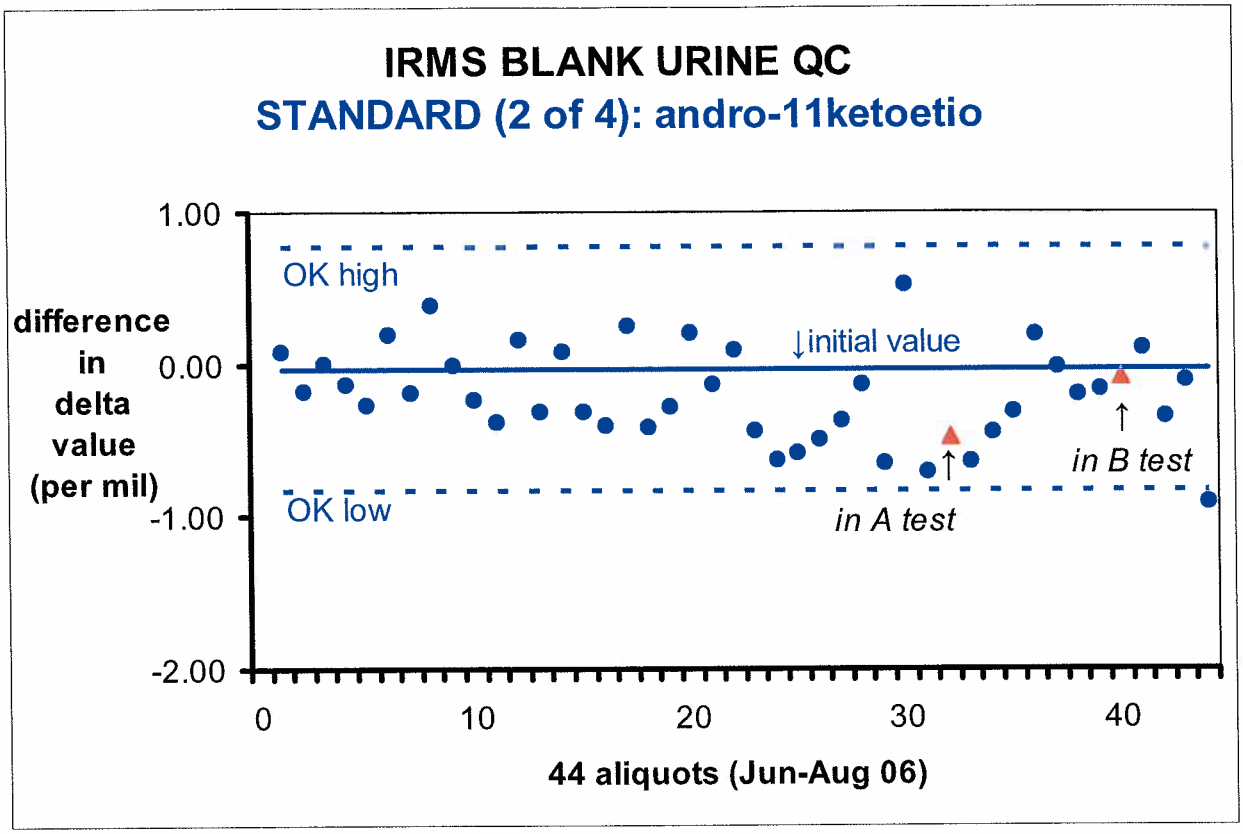

IRMS BLANK URINE QC
STANDARD (2 of 4): andro-11ketoetio
difference in delta value (per mil)
1.00
0.00
-1.00
-2.00
OK high
OK low
↓initial value
in A test
in B test
0
10
20
30
40
44 aliquots (Jun-Aug 06)

Figure 14

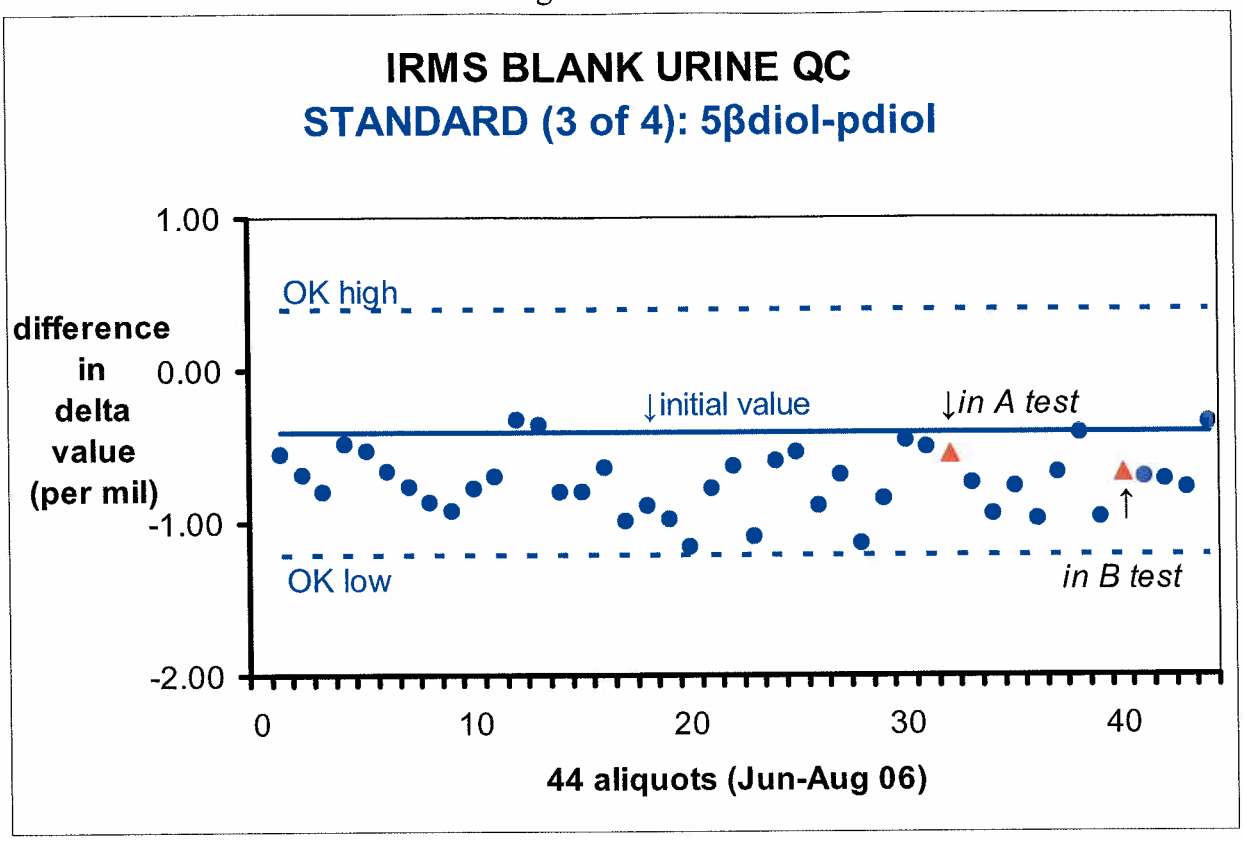

IRMS BLANK URINE QC
STANDARD (3 of 4): 5βdiol-pdiol
1.00
0.00
-1.00
-2.00
difference in delta value (per mil)
OK high
OK low
↓initial value
↓in A test
in B test
0
10
20
30
40
44 aliquots (Jun-Aug 06)

Figure 15

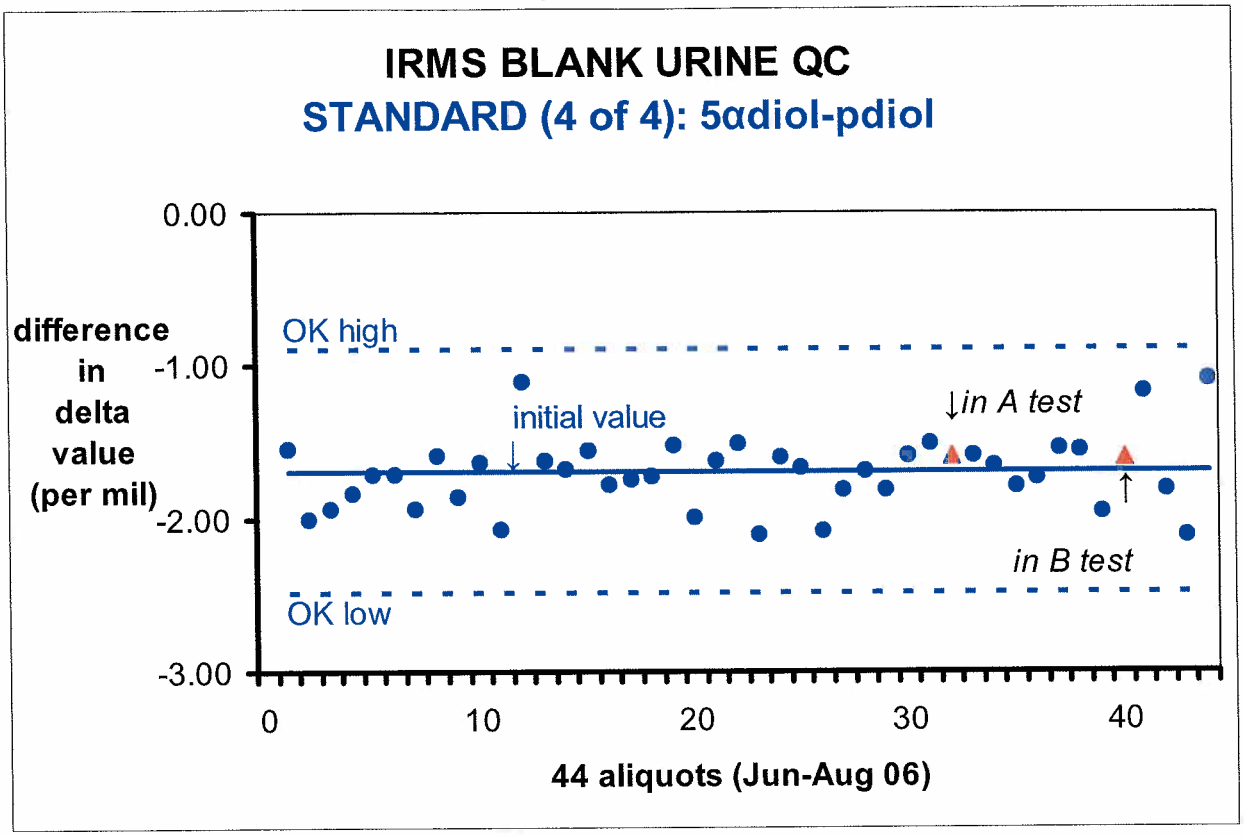

IRMS BLANK URINE QC
STANDARD (4 of 4): 5αdiol-pdiol
0.00
-1.00
-2.00
-3.00
difference in delta value (per mil)
OK high
OK low
initial value
in A test
in B test
0
10
20
30
40
44 aliquots (Jun-Aug 06)

Figure 16

Delta/Delta	Respondent	UCLA Subject
5 alpha diol-Pdiol	A -6.14 B -6.39	12-17 hours* -7.0**[1] 17-25 hours* -5.1**
5 beta diol-Pdiol	A -2.15 B -2.65	12-17 hours* -2.3** 17-25 hours* -2.3**

* after end of T administration

[1] **To render UCLA data comparable to Respondent's data, first the UCLA delta values were corrected for acetate as follows:

- UCLA acetylation reagent delta value: -37.35 (Exhibit ___, Table 2 footnote)

- UCLA corrected 5αdiol and 5βdiol delta value = (measured delta * 23 + 4 * 37.35)/19

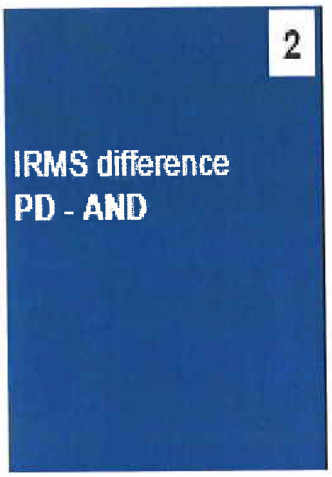
- UCLA corrected 5pdiol delta value = (measured delta * 25 + 4 * 37.35)/21

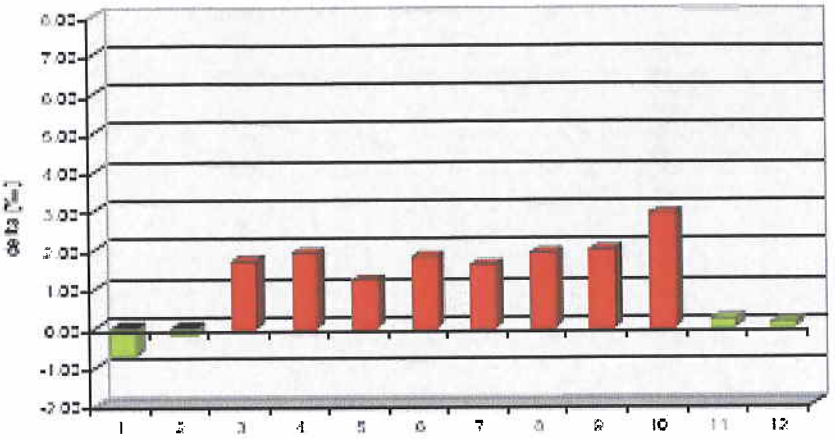
- then the delta/deltas were calculated based on the corrected deltas.

Figure 17

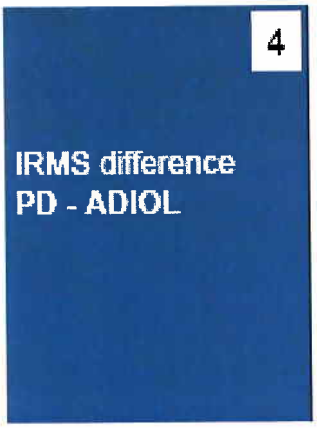
Volunteer P3

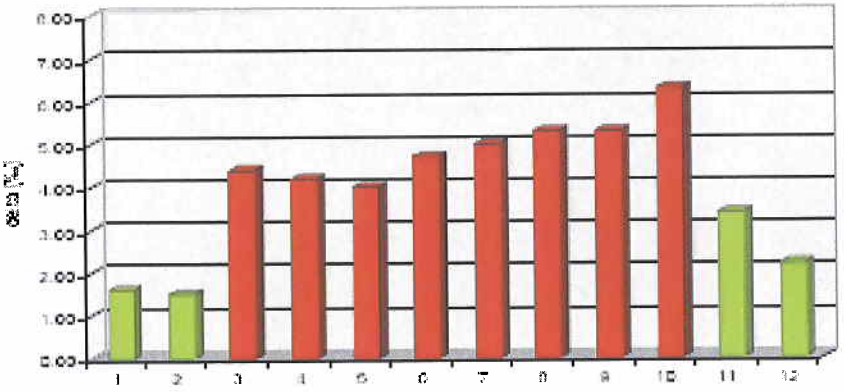
GC/C/IRMS results
continuos application of testosterone gel (P3)



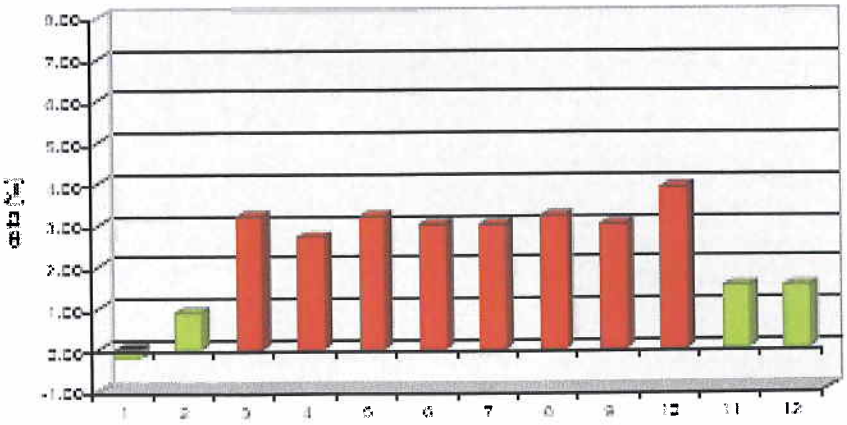


(Pdiol – Andro)









6

IRMS difference PD - ETIO

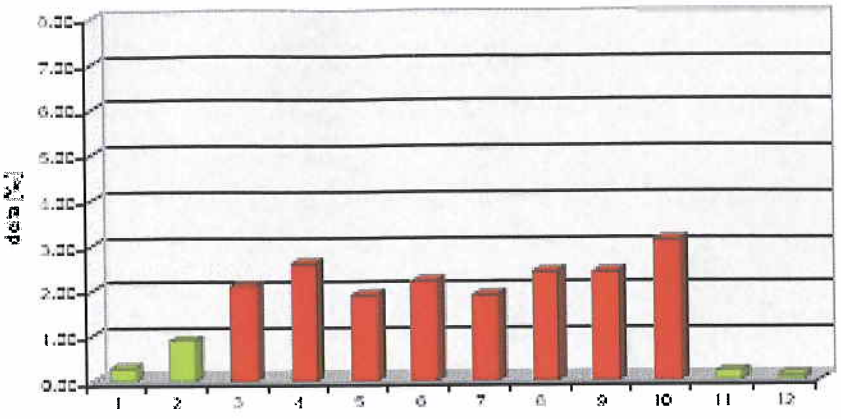
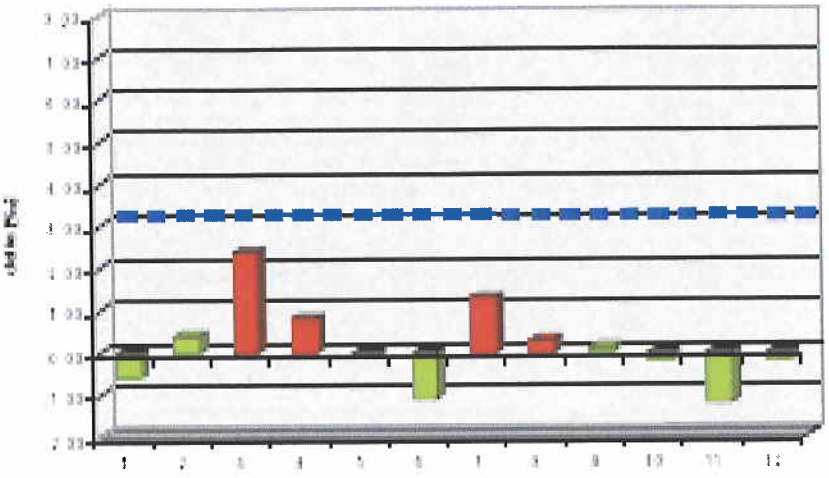


Figure 18

Volunteer P9

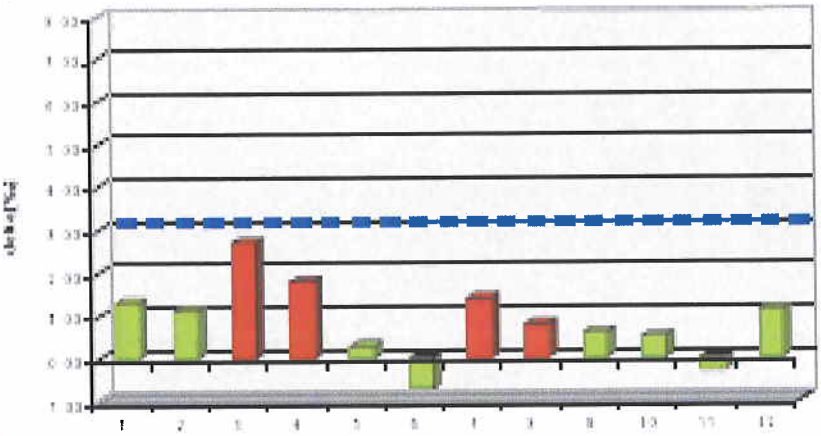
GC/C/IRMS results
intermittent application of testosterone gel (P9)

IRMS difference
11OHA – AND



(11OHA is one of the other endogenous reference compounds approved by WADA in TD2004EAAS (Exhibit ___, paragraph 3).)

IRMS difference
11OHA – BDIOL



IRMS difference
11OHA – ADIOL

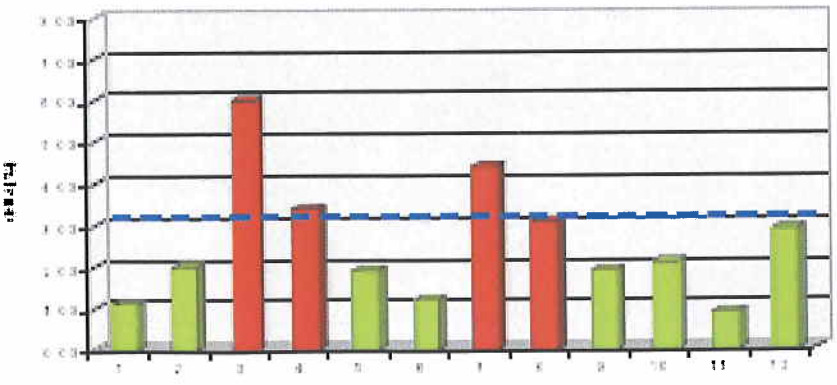
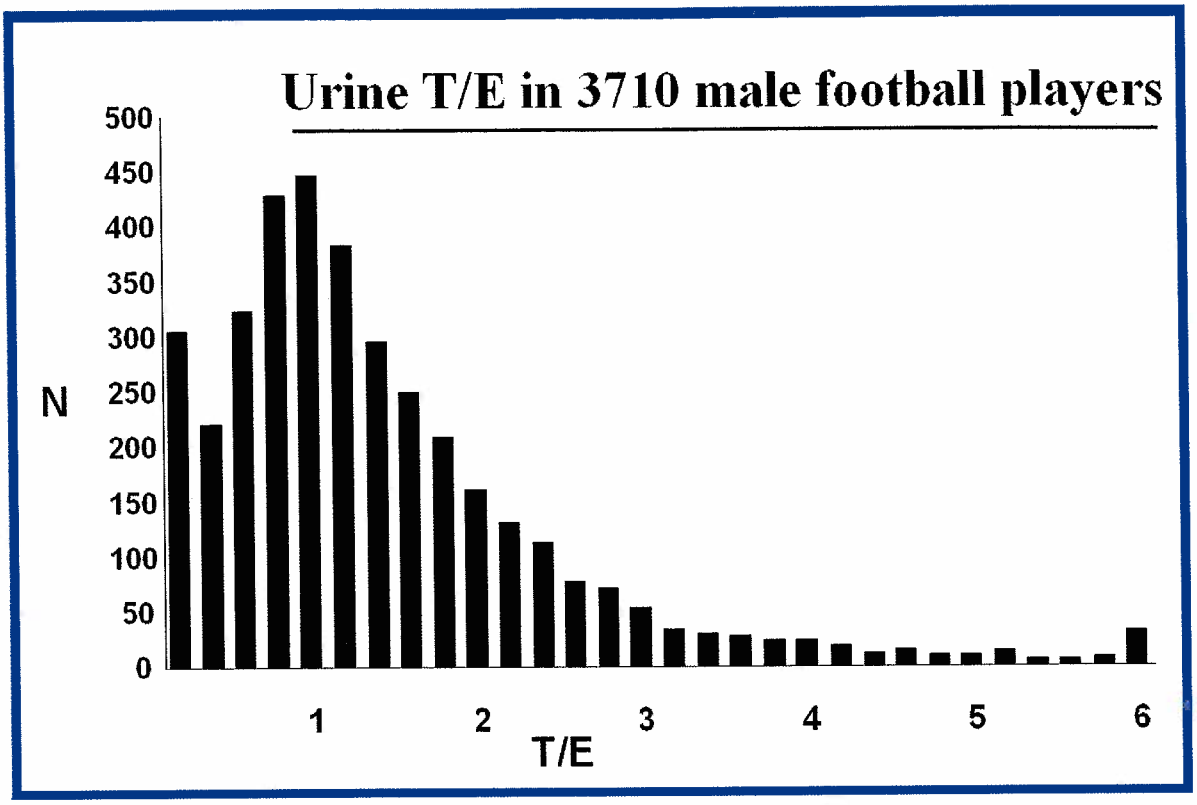


Figure 19



T/E <5.6 in 99% of subjects. *Issues in Detecting Abuse of Xenobiotic Anabolic Steroids and Testosterone by Analysis of Athletes' Urine* (Clin Chem, 43: 1280-1288, 1997)

Figure 20

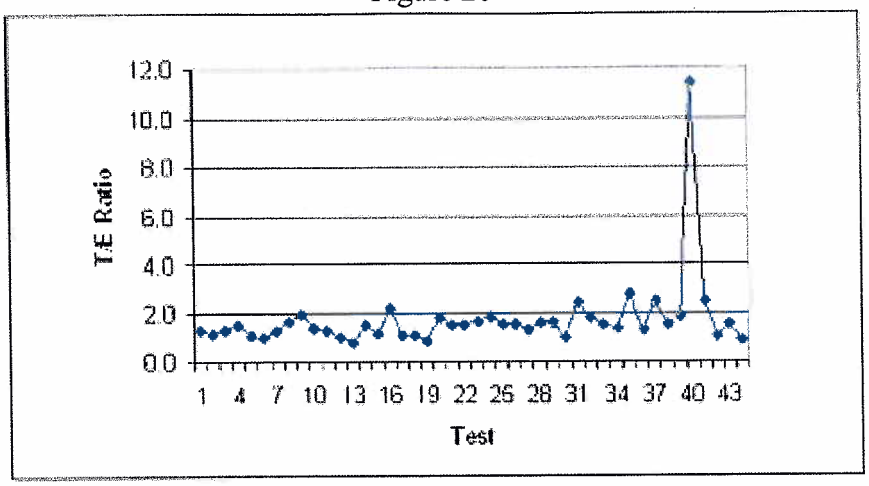

12.0
10.0
8.0
6.0
4.0
2.0
0.0
T:E Ratio
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43
Test

Figure 21. Consistency of "A" and "B" Confirmation T/E Ratios,
Testosterone and Epitestosterone Concentrations

Date	USADA #	Sample #	T/E Ratio	T Concentration	EpiT Concentration
July 24, '06	0093	178/07 995474	11.4	61.4	5.2
Aug 6, '06	0277-78	178/07 995474	10.9	63.2	5.9
	0279-80	178/07 995474	11.0	61.6	5.8
	0281-82	178/07 995474	11.1	60.2	5.6

TAB 39

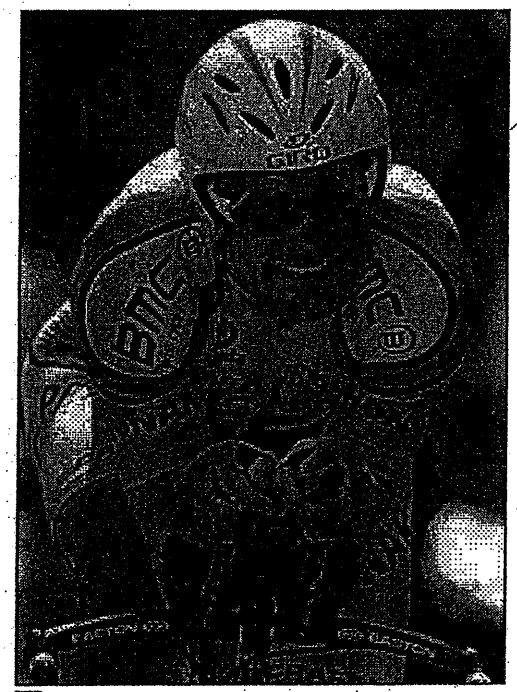
Search How do I find it?

Advertisement
Send your stress packing.
Pull back
FedEx

Synthetic testosterone found in Landis' 'B' sample; Tour winner likely to lose title

Updated 8/6/2006 11:26 PM ET

E-mail | Save | Print | Reprints & Permissions | Subscribe to stories like this RSS



Enlarge By Christian Hartmann, AP

Floyd Landis pedals his way to the Tour de France title last month.

By Sal Ruibal, USA TODAY

Floyd Landis remains optimistic despite Saturday's second positive doping sample from the Tour de France that triggered his removal as the event's 2006 champion.

He was also fired from his Phonak professional cycling team and will face a U.S. Anti-Doping Agency disciplinary hearing in three weeks that could result in a two-year suspension from racing.

"I have never taken any banned substance, including testosterone," Landis said Saturday morning in a prepared statement. "I was the strongest man in the Tour de France and that is why I am the champion. I will fight these charges with the same determination and intensity that I bring to my training and racing. It is now my goal to clear my name and restore what I worked so hard to achieve."

Landis was accused of having a testosterone-to-epitestosterone ratio above the allowed limits. A high ratio is believed to indicate the use of testosterone not produced by the body.

The head of France's anti-doping commission said the samples contained synthetic testosterone, indicating that it came from an outside source.

The second or "B" sample, "confirmed the result of an adverse analytical finding" in last week's "A" sample, the International Cycling Union said.

Pierre Bordry, who heads the French anti-doping council, said the lab that found higher-than-allowable levels of the hormone in both samples also discovered synthetic testosterone.

LATEST ON LANDIS

Landis lays out defense: Tour champ claims lab work was sloppy | Defense goes public

Jon Saraceno: Landis' excuses just don't add up | Jay Leno grills Landis on 'Tonight Show'



'B' sample: Synthetic testosterone found in second sample; Tour winner could lose title | **Q&A:** Doping expert explains testing process

Mike Lopresti: If Landis is guilty, he should confess | Drug test puts yet another hero in doubt



The simple man: Overwhelmed Landis trapped in complex problem



Sports Scope: Follow the latest on Landis and other cycling news

SPORTS SCOPE

More

THE SCREENING PROCESS

"I have received a text message from Chatenay-Malabry lab that indicates the 'B' sample of Floyd Landis' urine confirms testosterone was taken in an exogenous way," Bordry told The Associated Press.

Lab head Jacques De Ceaurriz said the isotope testing procedure was "foolproof."

"No error is possible in isotopic readings," he told the AP.

Landis had claimed the testosterone was "natural and produced by my own organism."

Landis hired Los Angeles attorney Howard Jacobs to represent him in the disciplinary process. Jacobs also represents cyclist Tyler Hamilton, who was accused of blood doping at the 2004 Summer Olympics — where he won a gold medal — and Tour of Spain.

Hamilton was cleared of the Olympics offense when his "B" sample was inadvertantly frozen. Both of his Tour of Spain samples were positive and he received a two-year suspension by USADA which was later upheld by the Court for Arbitration in Sport.

"At this point in time, I am waiting to receive the full laboratory documentation for the (Landis') "B" test," Jacobs said Saturday. "In consultation with some of the leading medical and scientific experts, we will prove that Floyd Landis's victory in the 2006 Tour de France was not aided in any respect by the use of any banned substances."

Landis and Jacobs will also argue against the UCI's premature release of the 'A' sample findings as well as the anonymous leak of the carbon-isotope test results to the *New York Times* on July 31.

"I call on the UCI to start following its own rules and to allow this process to proceed without the further taint of public comment by UCI officials," said Jacobs. "The anti-doping process must be free from the perception that sports federations and anti-doping authorities, who hold great political and financial sway over sport, are attempting to influence the outcome of a pending case by issuing inappropriate public comments."

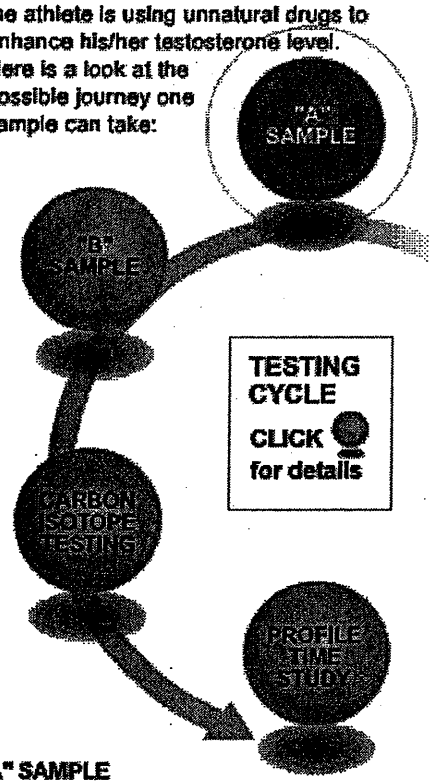
Landis is now in California and found out about the positive "B" sample through Internet wire reports.

"It has been a fight to keep up with the leaks," said spokesman Michael Henson. "Floyd is holding up well. He has a fire inside and wants to fight for the title that's rightfully his."

Phonak immediately severed ties with Landis

"Landis will be dismissed without notice for violating the team's internal Code of Ethics," Phonak said in a statement. "Landis will continue to have legal options to contest the findings.

The urine sample from an athlete can go through rigorous examination to verify if the athlete is using unnatural drugs to enhance his/her testosterone level. Here is a look at the possible journey one sample can take:



"A" SAMPLE

After each stage of the Tour de France, several riders give urine samples for subsequent drug screenings: the overall leader, the stage winner and up to three random competitors. Floyd Landis was the overall leader five times and the stage winner once, and has said he was tested at least six times during the race. The urine sample in question was taken after stage 17, the stage Landis made an incredible comeback to set up his victory. Like all samples, it was divided into an "A" sample and a "B" sample. The "A" sample is screened and the findings are reported while the "B" sample is saved and reserved in the event there is an abnormality discovered in the "A" sample. Screens are done on all samples to search for anything out of the ordinary, including the testosterone to epitestosterone (T/E) ratio. If the "A" sample shows a ratio of less than 4:1, the testing is finished. If the ratio is greater than 4:1, further testing is conducted.

Sources: Dr. Don Catlin, director of the Olympic Analytical Laboratory, UCLA; USA TODAY Research, AP

By Ron Coddington and Shawn Brouwer, USA TODAY

However, this will be his personal affair, and the Phonak team will no longer be involved in that."

Tour de France director Christian Prudhomme said Landis no longer was considered champion, but the decision to strip him of his title rests with the International Cycling Union.

"It goes without saying that for us Floyd Landis is no longer the winner of the 2006 Tour de France," Prudhomme told the AP in a telephone interview. "Our determination is even stronger now to fight against doping and to defend this magnificent sport."

Prudhomme said runner-up Oscar Pereiro of Spain would be the likely new winner.

"We can't imagine a different outcome," Prudhomme said.

If stripped of the title, Landis would become the first winner in the 103-year history of cycling's premier race to lose his Tour crown over doping allegations.

UCI lawyer Philippe Verbiest said Landis would officially remain Tour champion pending the American disciplinary process.

"Until he is found guilty or admits guilt, he will keep the yellow jersey," he said. "This is normal. You are not sanctioned before you are found guilty."

The results of the second test come nearly two weeks after he stood atop the winner's podium on the Champs-Elysees in the champion's yellow jersey.

Testosterone, a male sex hormone, helps build muscle and improve stamina. The urine tests were done July 20 after Landis' Stage 17 victory during a grueling Alpine leg, when he regained nearly eight minutes against then-leader Pereiro — and went on to win the three-week race.

The tests turned up a testosterone/epitestosterone ratio of 11:1 — far in excess of the 4:1 limit.

"It's incredibly disappointing," three-time Tour winner Greg LeMond told the Associated Press by phone from the starting line at the Pan Mass Challenge in Sturbridge, Mass. "I don't think he has much chance at all to try to prove his innocence."

"It doesn't end here," said Landis' Spanish lawyer, Jose Maria Buxeda. "What matters is the concept. A prohibited substance has been found in the samples, but no immediate sanction comes into effect yet. The rider will defend himself."

Landis, a 30-year-old former mountain biker, says he was tested eight other times during the three-week tour and those results came back negative.

Since the Phonak team was informed of the positive test on July 27, Landis and his defense team have offered

varying explanations for the high testosterone reading — including cortisone shots taken for pain in Landis' degenerating hip; drinking beer and whiskey the night before; thyroid medication; and his natural metabolism.

Another theory — dehydration — was rebuffed by anti-doping experts.

"When I heard it was synthetic hormone, it is almost impossible to be caused by natural events. It's kind of a downer," said LeMond, the first American to win the Tour. "I feel for Floyd's family. I hope Floyd will come clean on it and help the sport. We need to figure out how to clean the sport up, and we need the help of Floyd."

Contributing: Wire reports

Posted 8/4/2006 2:25 PM ET

Updated 8/6/2006 11:26 PM ET

E-mail | Save | Print | Reprints & Permissions | Subscribe to stories like this RSS

Free Callaway Golf Package
Free Callawy Golf Club Set with free shipping! Participate Now!
sports-fitness-rewardpath.com

Free Tiger Woods Shoes
Free Nike SP-7 Tiger Woods Shoes-Participate Now & Get It Free!
www.sports-offer.com

Hybrid Iron Set 1 thru PW, GW, and SW
Full set of hybrid irons, custom made for men, seniors, and ladies. Free online...
thomasgolf.com

Buy a link here

Advertisement



Newspaper Home Delivery - Subscribe Today

Copyright 2007 USA TODAY, a division of Gannett Co. Inc.

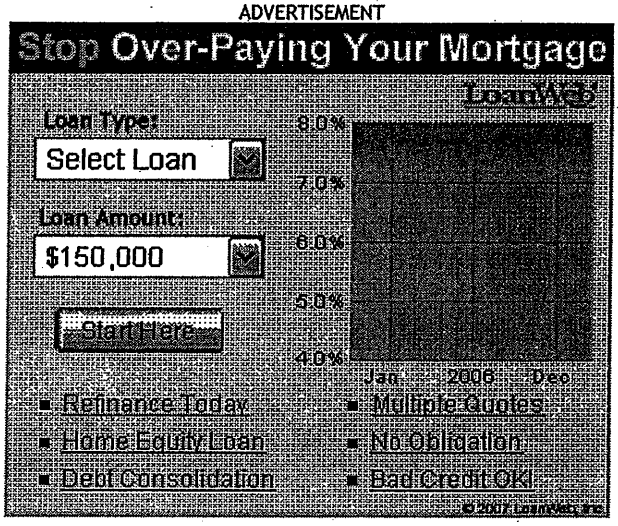
Tour de France Champ Floyd Landis Requests Test of Backup Urine Sample in Doping Flap

Monday , July 31, 2006

Associated Press

MADRID, Spain —

ADVERTISEMENT



Tour de France champion Floyd Landis officially requested the testing of his backup urine sample Monday in an effort to clear his name of doping allegations.

"We've send the fax to the UCI this afternoon," said Jose Maria Buxeda, one of Landis's two Spanish lawyers. "Now we'll have to adjust to their calendar."

The UCI is the Swiss-based international cycling union.

• For complete sports coverage visit FOXSports.com.

Buxeda said he's not sure when the "B" test will be carried out at the Paris doping laboratory, though it could be sometime this week.

Landis, who has returned to the United States, is willing to attend the analysis depending on the date, Buxeda said.

Landis tested positive for elevated testosterone levels following the 17th stage of the Tour de France, where he made a stunning solo breakaway in the Alps to put himself back into contention for victory after a poor performance the previous day.

If his the B test is negative, Landis would be cleared. If it's positive, the 30-year-old American would face formal doping charges and could be stripped of his Tour victory and banned for two years.

Landis's lawyers say they fully expect the backup test to confirm the original finding.

Landis, speaking in Madrid on Friday, said his test results had nothing to do with doping, and that the high level of testosterone in his body was the result of his natural physiology.

Testosterone is a naturally occurring male hormone that is banned when it is found in a ratio greater than 4:1 to another hormone, epitestosterone.

Former professional Spanish cyclist Jesus Manzano said he was given testosterone when he competed for the Kelme team, and immediately felt its effects.

"It gives you a lot of strength, and it works very well," he wrote in the Spanish sports daily AS on Saturday. "It produces a euphoria."

Oscar Pereiro of Spain, who finished second overall in the Tour de France, would be declared the winner if Landis loses the Tour de France title.

It would be the first time in the history of the Tour of France that the winner has been disqualified for doping.

Visit FOXSports.com for complete sports coverage.

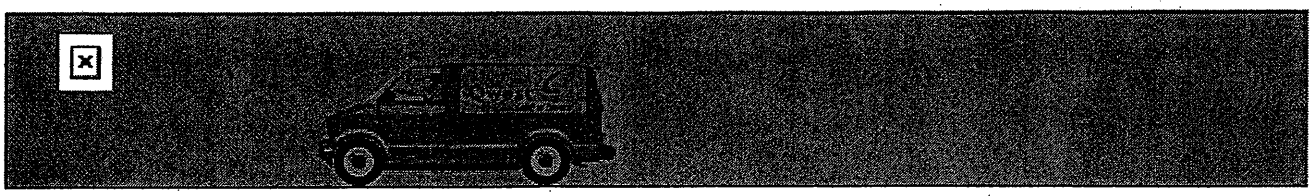
SEARCH GO

Click here for FOX News RSS Feeds

Advertise on FOX News Channel, FOXNews.com and FOX News Radio
Jobs at FOX News Channel.
Internships at FOX News Channel (now accepting Summer interns).
Terms of use. Privacy Statement. For FOXNews.com comments write to foxnewsonline@foxnews.com; For FOX News Channel comments write to comments@foxnews.com
© Associated Press. All rights reserved.
This material may not be published, broadcast, rewritten, or redistributed.

Copyright 2007 FOX News Network, LLC. All rights reserved.
All market data delayed 20 minutes.

Advertisement



Powered by

Landis fires back at cycling hierachy

Advertisement

Updated 8/6/2006 11:24 PM ET

By Sal Ruibal, USA TODAY

Embattled Tour de France champion Floyd Landis says he's been treated unfairly by cycling's international governing body and can't properly defend himself against charges he used illegal doping products to win the race.

In his first interview after his second, or "B" urine sample was reported Saturday to be positive for synthetic testosterone and an out-of-limits testosterone-to-epitestosterone level, Landis lashed out at officials of the Union Cycliste Internationale (UCI) and the World Anti-Doping Agency for giving information on his case to the media while not providing him with reports on his test results.

The results of his initial test were made public July 27.

"I just got the information on the 'A' sample a day and half ago," he said. "I had to find out about the 'B' from reading it in the media."

Landis also pointed out that American sprinter Justin Gatlin's positive "A" test for an illegal testosterone ratio was not announced by track and field officials for three months, "while I had only two days to react to mine. (UCI head) Pat McQuaid said he had to release mine before the lab leaked it."

He also pointed out that his remarkable comeback performance on Stage 17 — the stage where his positive tests came from — was not an anomaly, but his doping tests were.

"I put in more than 20,000 kilometers of training for the Tour. I won the Tour of California, Paris-Nice and the Tour de Georgia. I was tested eight times at the Tour (de France); four times before that stage and three times after, including three blood tests. Only one came back positive.

"Nobody in their right mind would take testosterone just once; it doesn't work that way."

The 30-year-old racer says the biggest mistake he did make was responding to media reports as they surfaced, giving the impression he was coming up with new excuses daily.

"I've been catching a lot of grief in the press: 'Floyd has a new excuse, a new reason for what happened.' This is a situation where I'm forced to defend myself in the media. It would never have happened if UCI and WADA had followed their own rules," Landis said.

"There's some kind of agenda there," he said wearily. "I just don't know what it is."

Describing himself as "reasonable and logical," Landis refuses to see himself as a victim in the case and says there's "zero chance" someone from his Phonak team either inadvertently or purposely gave him synthetic testosterone.

Landis will undergo hip replacement surgery in about two weeks, then prepare for a hearing before the U.S. Anti-Doping Agency sometime in the next month. He has not received notice from USADA.

"Something bad happened to me," he says, "but bicycle racing is the most beautiful sport in the world. I want to remain part of it."

He longs to ride his bike but says that is impossible because of the media crush around his home.

"I need to (ride), believe me," he said. "That will be the best day of the week if that happens."

Find this article at:
http://www.usatoday.com/sports/cycling/2006-08-06-landis-retort_x.htm

☐ Check the box to include the list of links referenced in the article.

Related Advertising Links

Scottrade Online Broker
$7 online trades. Fast, accurate executions. 285+ offices nationwide.
www.scottrade.com

Refinance Rates at 5.5% Fixed!
$300,000 mortgage for under $719/mo. Get 4 free quotes. Bad credit ok!
www.fetchArate.com

Calculate Your New Mortgage Pa
$310K loan for under $998/mo. Thir pay too much? Refi now.
www.lowermybills.com

Place your ad here

TAB 40

C. Ayotte, A. Charlebois, S. Lapointe, D. Barriault and M. Sylvestre

Validity of urine samples : microbial degradation

INRS-Santé, Montréal, Québec

Since several years, most often during summertime, we have observed the complete degradation of some urine samples upon arrival at the laboratory. Whatever conservation methods are used in the laboratory, the damages are irreversible and some analytical results are invalid.

The collection of the urine samples is not done under sterile conditions and it is possible that micro-organisms be present and multiply during transportation (delays) under favourable conditions and improper storage. Preliminary studies have permitted in some of these specimens, the identification of strains of *Enterobacteria* and *Staphylococcus*, but not in all, the micro-organisms being already dead. These micro-organisms are found in the gut microflora or on the skin, their presence in urine samples collected in these conditions is no surprise. However, several specimens contaminated by such microbes were not showing signs of degradation. On the other hand, contamination may occur in the laboratory namely from the water used. In the past years, some experts have raised the possibility of microbial formation of testosterone in positive athlete's urine sample to contest the validity of the laboratory findings[1]. Their claim is that enzymes such as the 3(17)-hydroxysteroid dehydrogenases (HSDH) and the Δ^4-5β-dehydrogenase for example, are able to convert several steroids to testosterone and that such enzymes are widely found in the micro-organisms that can contaminate urine samples. Some potential sources of microbial testosterone were described in the sixties. For example, the conversion of 5β-steroid to Δ^4-steroid by the steroid Δ^4-5β-dehydrogenase was reported[2] and 3β-HSDH and Δ^4 nuclear steroid dehydrogenase may convert saturated steroid to testosterone *via* 4-androsten-3,17-dione and 1,4-androstadiendione[3].

[1] 1994-1995 Cases : D. Modahl, UK, A. Okeke, Norway, M. Gravelle, USA ; R.S. Bilton, *Lancet(letter)*, 345 (1995) 1186

[2] S.J. Davidson and P. Talalay et al., JBC, 241 (1966) p.906; Enzyme Handbook, T.E. Barman, Donnelley & Sons, Virginia, USA, (1969) p. 166.

[3] R. S. Bilton *Lancet*, 345 (1995) 1186, Enzyme Handbook, T.E. Barman ed. (1969), P. Talalay et al. JBC, 218 (1956) 675.

USADA1154

We have shown from preliminary work and after detailed analysis of the urinary steroids, that when some contaminated samples were subjected to temperatures of 37°C during days, the steroid conjugates were hydrolysed and testosterone and other urinary steroids accumulated in the free fraction[4](figure 1). Although we have not observed the alteration of the structure of the steroids (figure 2), the analysis of testosterone in the conjugated fraction was invalid. The increase of free testosterone in urine samples was also reported by Kjeld et al.[5]. The stability of steroids conjugates such as testosterone glucuronide in urine matrix was not studied extensively but it was shown and it is widely accepted, that their measurement is not impaired when the samples are stored at -20°C for up to one year[6]. We have observed that if the urine samples are not contaminated, the steroid glucuronides are stable under these conditions, while the more labile steroid sulfates such as epiandrosterone, androstendiol, dehydroepiandrosterone are hydrolysed to a certain extend.

In the presence of other micro-organisms, the effects were more important and cumulative(figure 3). In the cases observed, the major urinary steroids such as androsterone, etiocholanolone, epiandrosterone, testosterone and the androstandiols were extensively degraded and other steroids were formed and accumulated in the free fraction. These steroids were identified as the 5α- and 5β-androstan-3,17-dione (figure 4) usually present in trace amount in urine samples. After a several days, the natural steroids are undetectable (figure 5). The formation of 5α-androstandione which may hide the epitestosterone combined with the degradation of testosterone and epitestosterone caused an apparent reduction of the T/E value in some chromatographic systems[7](figure 6), DHT is an intermediate in the microbial conversion of androstandiol to androstandione. Under such conditions, the analysis of natural steroids such as testosterone, epitestosterone and DHT is invalid. Since early 1991, our screening procedure includes the detection of the androstandiones and urine specimens showing their presence are given as invalid.

[4] C. Ayotte, A. Charlebois and D. Goudreault, *J. Chromatogr., Biomed. Appl.*, accepted for publication in January 1995

[5] J.M. Kjeld, C.M. Puah and G.F. Joplin, *Clin. Chim. Acta*, 80 (1977) 285

[6] K. Robards and P. Towers, *Biomed. Chromatogr.*, 4 (1990) 1 ; E. Venturelli, A. Cavalleri and G. Secreto, . *Chromatogr., Biomed. Appl.*, 671 (1995) 363

[7] CAP revision report (1992)[7]

USADA1155

Similar effects can be observed during the enzymatic hydrolysis. Some crude mixtures of *H. pomatia* juices are known to possess some unwanted enzymatic activities causing the degradation of the urinary steroids[8]. When confirming an elevated T/E value, the free fraction is now also analysed in order to verify the absence of testosterone in the free form. Although it was previously reported[9], we never observed the formation of free testosterone in urine samples ; however, more systematic investigations will be done. Furthermore, the measure of an elevated urinary pH value, although indicating the ageing of the sample, cannot in itself invalidate the analysis of a sample. Only half of the specimens of very high pH values (between 8 and 9) have shown signs of degradation invalidating the measure of the T/E value (figure 7) . Conversely, some heavily degraded samples were showing normal pH values.

The formation of the 5α/β-androstandiones or the deconjugation of the urinary steroids are not observed when samples are stored at -20°C to 4°C even for prolonged periods of time. Both reactions are inhibited by bacteriostatic agents such as sodium azide and mercuric chloride added in concentrations of 0.5%. The agents must be added few hours before the incubation to effectively precipitate proteins. Lowering the pH is not necessary and may cause the hydrolysis of the steroids sulfates such as $HgCl_2$ does.

Conclusion

We are now investigating more completely the bacterial contamination of urine samples. We will make a repertoire of the microbes found in specimens and of their effects. Inoculations of sterile urine samples and of culture media containing authentic steroids standards should provide more definitive answer, especially concerning the real possibility of forming testosterone in the samples. However, at this moment, urine samples collected under the present conditions are very often contaminated by microbes but this will not necessarily lead to the degradation of the specimen. Both degradation processes are stopped when samples are stored at temperatures below -4°C. Improvement of the transportation to the laboratory can solve the problem since even at 37°C, nearly four days are needed to observe the signs of degradation. Instructions must also be given to

[8] M. Donike et al., R. Massé, C. Ayotte and R. Dugal, *J. Chromatogr.*, 489 (1989) 23 ; E. Vanluchene, W. Eechaute and D. Vandekerckhove, *J. Steroid Biochem.*, 16 (1982) 701

[9] H. Geyer et al., Proceedings of the 13th Workshop on Dope Analysis, (1996) p.95.

USADA1156

doping control officers to insure a proper refrigeration of the specimens and this may prevent the addition of a bacteriostatic agent. The laboratory should report the results as invalid when signs of degradation are present.

USADA1157

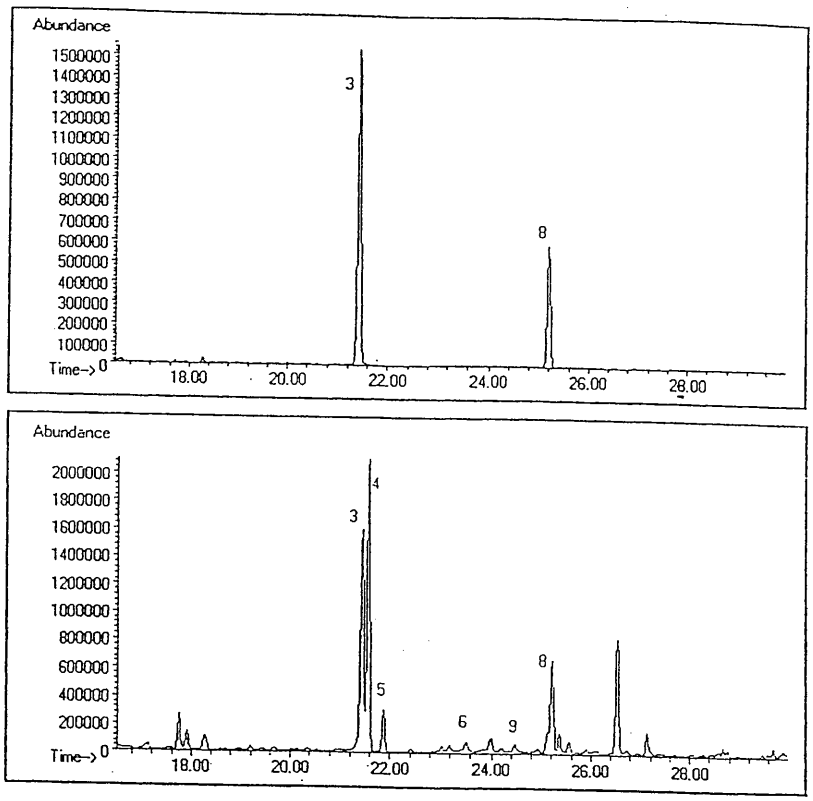


Fig. 1 - Free fraction of a urine sample contaminated by bacteria (not identified) and incubated at 37°C for 24 hours (upper panel) and 120 hours (lower panel). Peak 3 : epietiocholanolone (ISTD), 4 : androsterone and etiocholanolone, 5 : 5α, ß-androstan-3α, 17ß-diol, 6 : epitestostreone, 8 : ISTD, 9 : testosterone

USADA1158

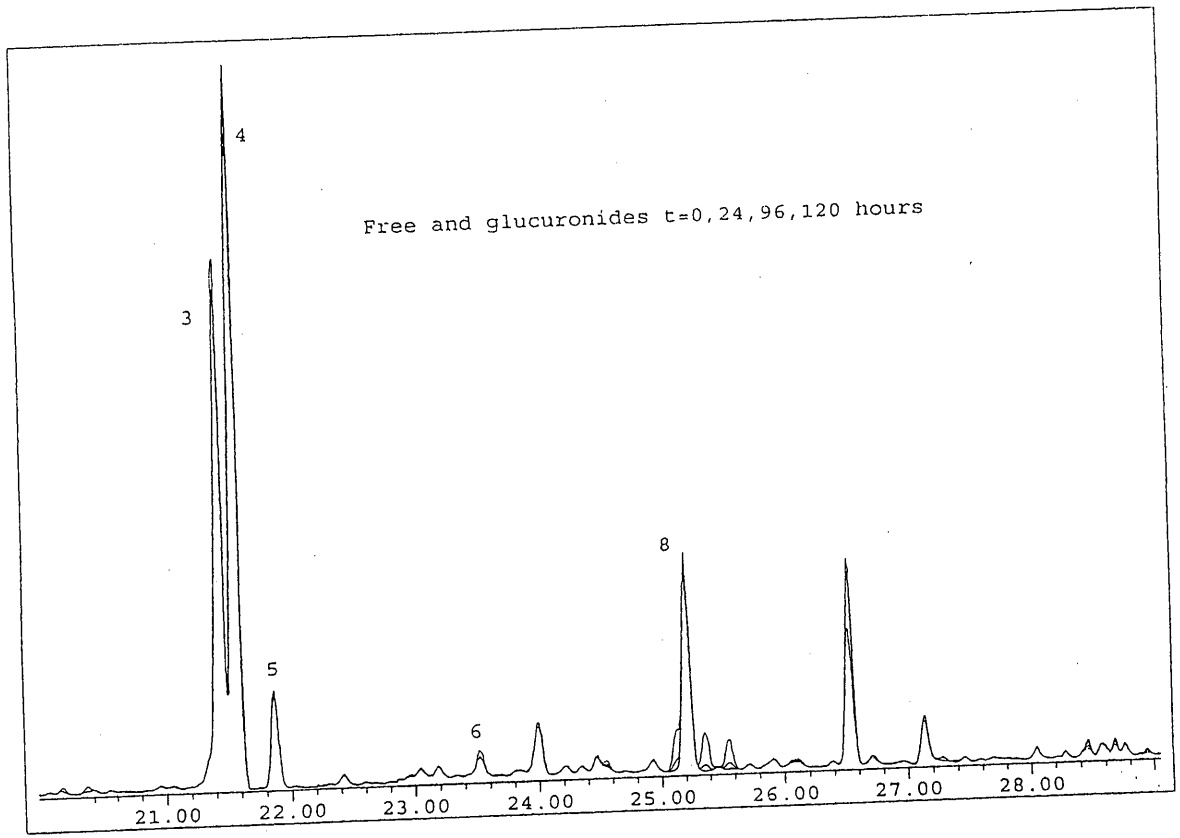


Fig. 2 - Urine sample contaminated by bacteria (not identified) and incubated at 37°C.

USADA1159

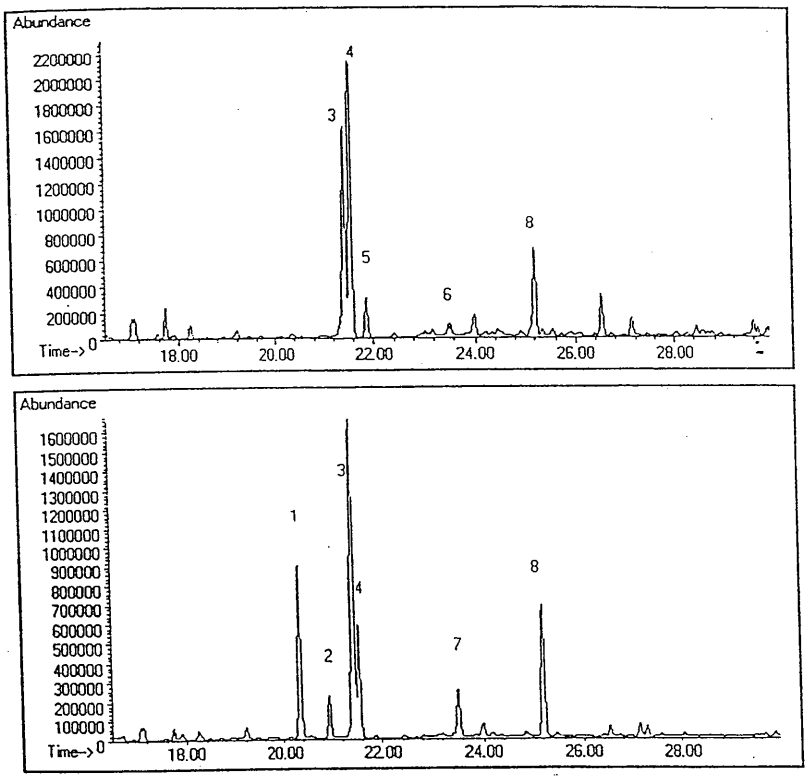


Fig. 3 - Urine sample inoculated with bacteria (not identified) (free and glucuronide fractions) and incubated at 37°C for 6 hours (upper panel) and 120 hours (lower panel). Peaks 1, 2 : 5ß-androstan-3,17-dione, 3 : epietiocholanolone (ISTD), 4 : A and Etio, 5 : 5α,ß-androstan-3α,17ß-diols, 6 : epitestosterone, 7 : 5α-androstan-3,17-dione, 8 : ISTD

USADA1160

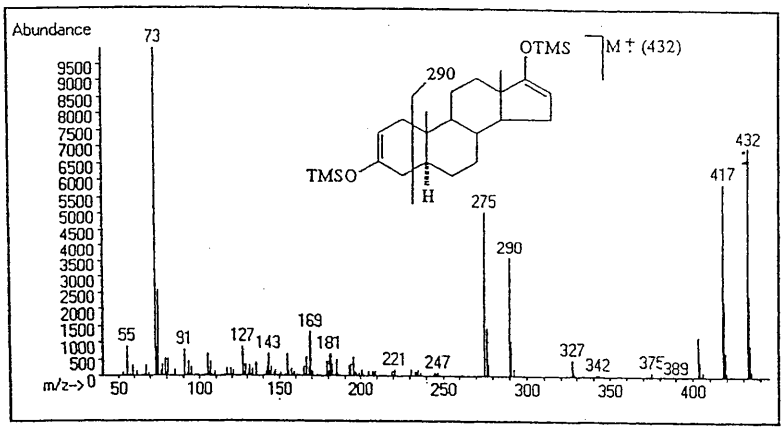


Fig. 4 - Mass spectrum of the TMS-enol derivative of 5α-androstan-3,17-dione obtained from the GC/MS analysis of the authentic standard (20 ng injected).

USADA1161

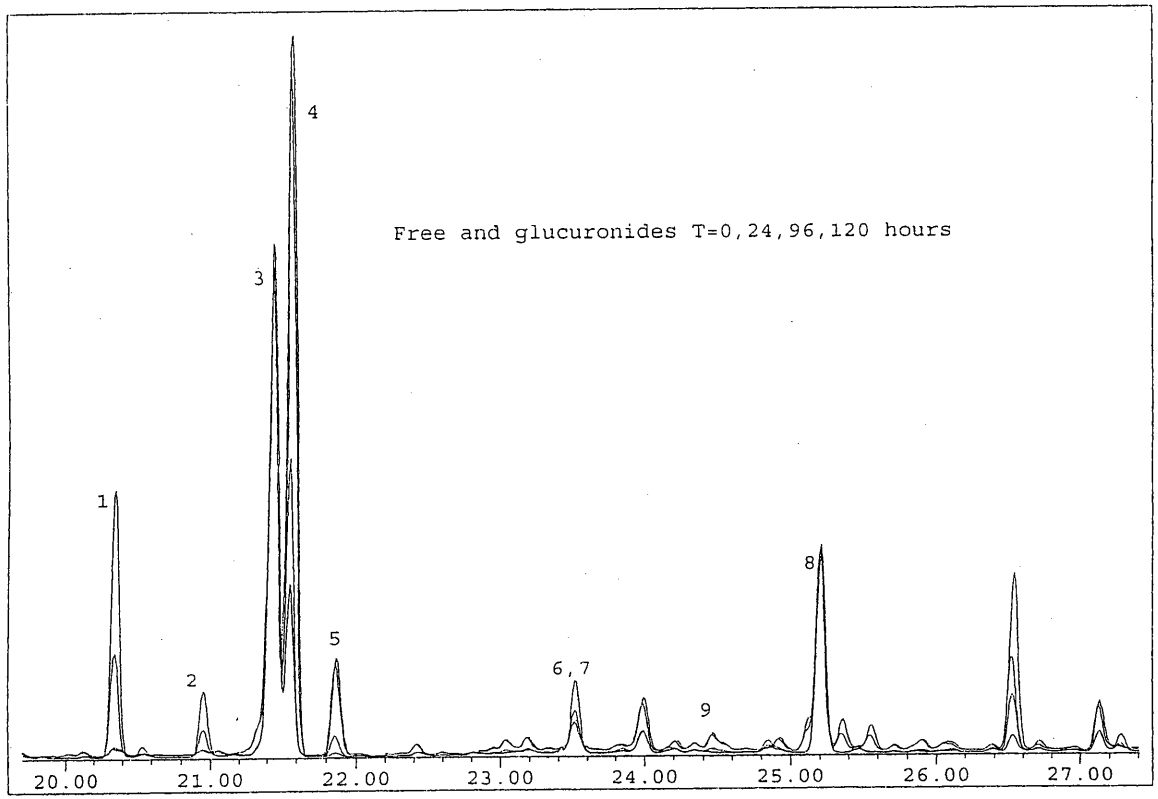


Fig. 5 - Urine sample (50 mL) inoculated with 1.0 mL of a sample containing bacteria. Incubations done at 37°C.

USADA1162

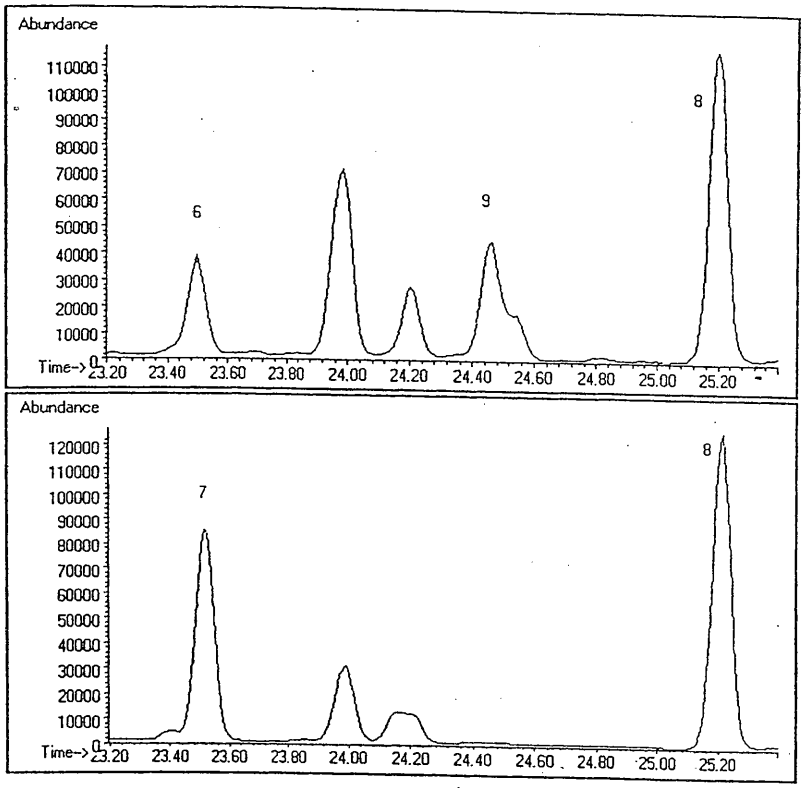


Fig. 6 - GC/MS analysis (432.4 and 435.4 ion chromatograms) of the free and glucuroconjugated fractions of a urine sample contaminated by bacteria, 6 hours after inoculation (upper panel) and 120 hours after (lower panel). Peaks 6 : epitestosterone, 7 : 5α-androstan-3,17-dione, 8 : 17α-methyl-5α-androstan-3ß,17ß-diol (internal standard), 9 : testosterone.

USADA1163

USADA1164

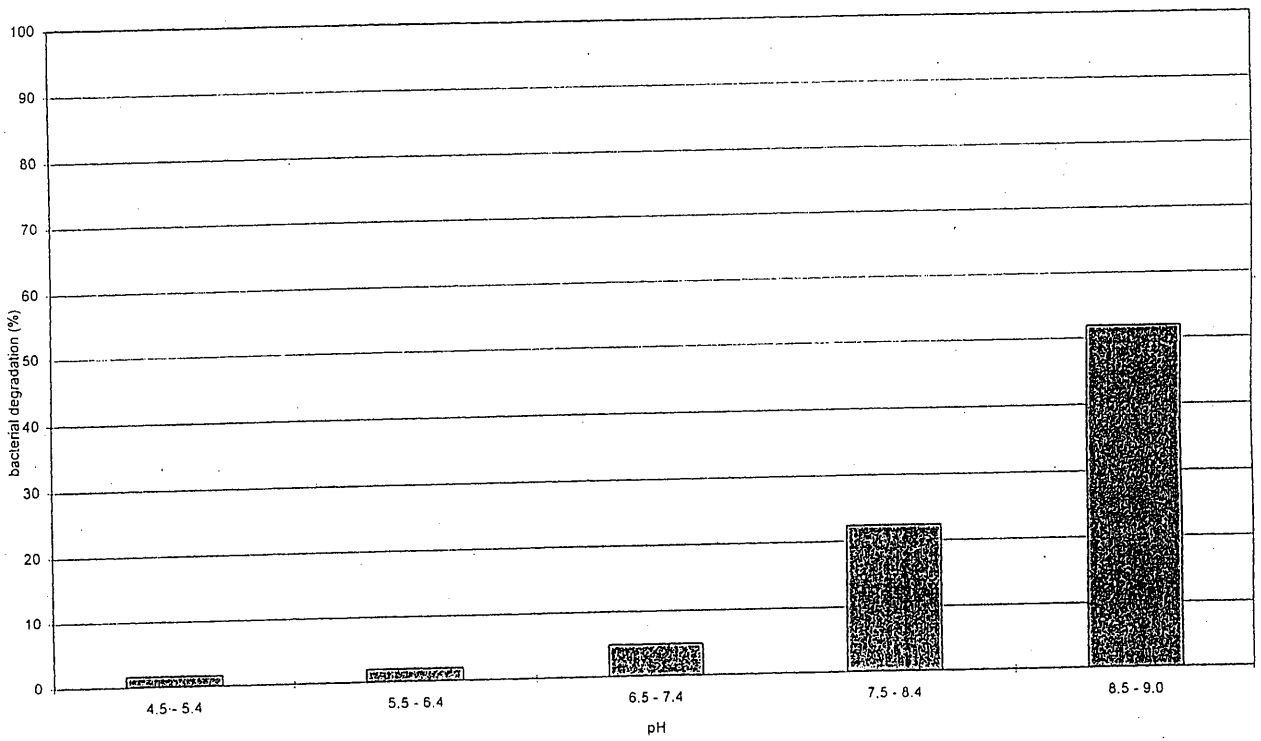


Fig. 7 - Percentage of samples showing signs of bacterial degradation in relation with the pH values of specimens received from doping control programs (1994-1995).

USADA1165

J. Baenziger and L. Bowers

Variability of T/E Ratios in Athletes

Sports Medicine Drug Identification Laboratory, Department of Pathology and Laboratory Medicine, Indiana University Medical Center, Indianapolis, Indiana, USA

INTRODUCTION:

The use of the Testosterone(T)/Epitestosterone(E) Ratio (T/E) has been the primary method used to determine whether an athlete has used exogenous testosterone. The finding of T/E results greater than 6:1 has been considered as a positive anti-doping test by the IOC since 1982. Since this time a few athletes were identified which had elevated T/E ratios and after extensive evaluation this elevated T/E ratio was considered to be due to a normal physiologic variation. Many athletic programs will now more frequently test those individuals who have a T/E ratio between 6-10:1 before considering taking punitive actions[1]. However, it is also known that some athletes are using exogenous testosterone or other manipulations of the endocrine system by various substances, yet their T/E ratios never exceed 6:1. Several methods are being investigated to help resolve the above problems[2] , and we now present preliminary data on the use of T/E Ratio variability as a tool to help resolve some of these problems.

SUBJECTS:

Urine samples were collected from a group of male athletes (subjects) as part of their athletic organization's Anti-doping Program during 1990-1991. Multiple samples from the subjects were received over this time period and were uniquely identified by a sample number so that the analyses of the T/E ratios were performed blind to the relationship between samples. The actual identity of the subject is only known to the athletic organization with each subject having a unique player number assigned. Subsequent to the analysis, the athletic organization generated a list of sample numbers that were associated

with the unique player number for our use in matching sample results for each subject.

METHODS:

Anabolic Steroid Analysis:

The T/E Ratio was determined on each subject's sample(s) using our laboratory's routine procedure for conjugated steroids[3]. In brief, 5 ml of urine is extracted using a C-18 solid phase column. The column is eluted with methanol, evaporated, and then hydrolyzed using Helix pomatia. The hydrolysate is extracted with ether and then derivitized with MSTFA/TMIS to silinate all hydroxyl and keto functions. Analysis is performed using one of 5 Hewlett/Packard 5890 GC's equipped with a capillary column and attached to 5970B MSD detectors operated in the SIM mode. The T/E ratio's were determined by comparing the peak area of testosterone and epitestosterone using the $M^{+\bullet}$ (432 m/z) ion signal. Additional analytical data from endogenous urinary steroids is also routinely collected and recorded along with other sample data as follows:

Specimen Data	Analytical Data
Subject's specimen ID#	Androsterone(A) & Etiocholanolone(Et) areas
Collection date of specimen	11-ßOH A & Et areas
Receipt date of specimen	Testosterone & Epitestosterone areas
Subject's sport	Internal Standard area
Subject's sex	Creatinine concentration
Laboratory Accession #	Specific gravity
Laboratory Batch #	Urine pH

Within each analytical batch analysis (20 subject samples) the following controls and standards are simultaneously analyzed: a conjugated control (both abnormal and normal); plus an endogenous standard which includes A, Et, 11-OH A, 11-OH Et, T, and E. Thus, semi-quantitative concentrations for the endogenous steroids can be determined on each subject by comparing area ratios for each steroid with the endogenous standard. These concentrations are currently being entered into a comprehensive subject database.

Data Evaluation:

The results of the T/E ratio determinations for each subject were evaluated and the mean, standard error of the mean (SEM), standard deviation (SD), percent coefficient of variation (%CV), and percent standard error of the mean (%SEM) were calculated using routine statistical methods. A subset of subjects were selected for further evaluation of T/E variability and consisted of those subjects who had at least one T/E result greater than 3 and who had at least 3 samples analyzed.

RESULTS:

Analytical Performance:

The analytical precision of the T/E ratio for the screening procedure has been determined by recording the T/E ratio from both the abnormal (mean = 7.4, %CV = 8) and normal (mean = 1.4, %CV = 15) conjugated controls over multiple batch assays and across 5 instruments.

Subject Population Characteristics:

A total of 1285 subjects were tested with 4973 T/E results measured. During this same time period a total of 22,806 T/E ratios were performed on athletes from all sports in the laboratory. The two populations' T/E ratios are compared in Table 1 and Figures 1 and 2, which show that the two populations are similar.

The mean T/E was determined in a subset of 796 subjects who had at least 3 values recorded and compared to the mean of the 1285 subjects. These populations' T/E ratios are compared in Table 1 and in Figures 3 and 4, which again show that the populations are similar. The populations are further characterized by determining the values at the 90th, 95th, and 99th percentiles for these non-gaussian population distributions.

Subject Variability of T/E Ratios:

The variability of each subject's T/E determinations were calculated on the population subset of 796 subjects who had 3 or more values recorded. Figure 5 shows the distribution of the number of values that have been recorded for each subject. The results of these variability determinations are summarized in Tables 2-3 and in Figures 6-9. For each variability statistic a plot of the distribution frequency as well as a plot versus the mean T/E ratio was made. The variability determinations demonstrated a non-gaussian distribution and so the 90th, 95th, and 99th percentile values are listed in the Tables.

Subject Screening Procedure:

A second subset of the total 1285 subject population was selected on the basis of

USADA1166

USADA1167

whether a subject had at least one T/E result > 3:1 (approximately the 90th percentile for all athletes). This subset was further evaluated to determine the variability in their T/E results. A total of 122 subjects with 629 T/E measurements were identified using the above criteria. Of these 122 subjects, 97 subjects (8% of total subjects) also had at least 3 T/E values to use for variability calculations. These 97 subjects could be divided into three basic patterns of T/E variability:

Pattern Type 1: elevated T/E ratio but with low variability

Pattern Type 2: high variability as the result of a T/E "spike"

Pattern Type 3: high variability without a distinct T/E "spike"

Of these 97 subjects, 33 subjects had Pattern Type 1, 38 subjects had Pattern Type 2, 21 subjects had Pattern Type 3, and 5 subjects could not be classified into either Pattern 2 or 3. Graphical representations of a selected number of each Pattern Type are shown in Figures 10-12.

DISCUSSION/CONCLUSIONS:

These preliminary results of the variability of T/E ratios on this population of athlete subjects shows that the vast majority of subjects have low T/E ratios (ie clustered around the population mean of 1.2) and demonstrate little variability in their T/E ratios over time (ie %CV < 30%). Comparing the T/E variability (%CV) between the subjects and the analytical precision it is concluded that most of a subject's T/E variability is a determinate of the analytical imprecision. However, a small fraction of the subjects do show large variability in their T/E measurements over time and thus warrant additional evaluation.

The finding of a small fraction of subjects with relatively high T/E ratios (ie > 3:1) and with low variability (ie Pattern Type 1) may be due to a normal physiologic variation between subjects. In fact, our laboratory has documented several individuals who have had T/E ratios > 6:1 and who have demonstrated relatively consistent T/E ratios over several years and who have been further investigated using serum hormonal studies to document that the pituitary-testicular endocrine system is not suppressed or abnormal. The specific explanation for why T/E ratios may be elevated normally in some individuals is still not available, but research is currently underway that may help determine how this finding relates to an individual's production or metabolism of androgenic steroids.

The use by athletes of aqueous based testosterone with its relatively short half-life, epitestosterone use, and other techniques that alter the endocrine system makes timing of specimen collection critical to detect an elevated T/E ratio on a single determination. To detect this type of anabolic steroid misuse it has been suggested that the T/E ratio cut-off be lowered (ie 3:1) and that serial specimens be collected and used to evaluate the variability of an athlete's T/E measurements. The data presented in this study suggests that this may indeed be a viable method. These results indicate that the finding of an elevated T/E ratio (ie > 3:1) and a large T/E variability (ie > 30 %CV) is statistically uncommon in an athletic population. Thus those subjects with Pattern Type 3 are of interest since the large variability in T/E ratio results indicates variable excretion of T and E which has been seen in persons who are receiving exogenous aqueous testosterone or in persons whose pituitary-testicular endocrine system has been variably suppressed by anabolic steroids[4,5]. In addition, the finding of an isolated "T/E spike" as is seen in the Pattern Type 2 cases is strong evidence that exogenous testosterone use has occurred.

FUTURE PLANS:

Investigations are underway in various laboratories that are evaluating the endocrine and metabolic changes that result from the use of exogenous testosterone or other synthetic anabolic steroids. Donike[6] has described the use of urine "steroid profiles" to detect previous exogenous testosterone or anabolic steroid use and consists of comparing the relative quantities of various androgenic steroid metabolites in urine. Donike has found that the excretion of various androgenic steroids is altered in individuals who have used synthetic anabolic steroids or exogenous testosterone. This current study is presented as a preliminary evaluation of the intra-individual variability of T/E ratios in a large population. All data from these subjects are stored on tape and subsequent evaluation will consist of the determination of the quantitative levels of testosterone, epitestosterone, and other endogenous androgenic steroids present in each sample. Thus, a combined evaluation of both the "steroid profile" and T/E variability over time will be investigated, and may be of additional benefit for the detection of anabolic steroid doping. Additional samples from these subjects are continually being analyzed and thus future decoding of these specimens will allow for this database to be continuously evaluated over time.

Additional measurements of urinary luteinizing hormone (LH) levels, plus serum hormone determinations when available will also be incorporated into the on-going study of these subjects.

REFERENCES:

1. International Olympic Committee, List of Banned Substances, 1993.

2. Palonek E and Garle M. "Single Injection of Testosterone to 7 Volunteers: Results from this Study". Proceedings of 10th Cologne Workshop on Dope Analysis, June, 1992. ISBN 3-89001-011-3

3. Sample, RHB and Baenziger, JC. Gas Chromatography/Mass Spectrometric Detection of Anabolic Steroids. In, Analytical Aspects of Toxicology, edited by Deutsch, John R. Wiley and Sons, Inc., New York, 1989.

4. Donike M, Geyer H, Schanzer W, and Zimmerman J. "Die Suppression der endogenen Androgenproduktion durch Metandienon" in Training and Sport zur Pravention und Rehabilitation in der technisierten Umwelt Eds. I.-W. Franz, H. Mellerowicz, W. Noack. Springer Verlag Berlin-Heidelberg-New York-Tokyo. 1985.

5. Donike M, Geyer H, Kraft M and Rauth S. "Longterm Influence of Anabolic Steroid Misuse on the Steroid Profile" in Official Proceedings of the 2nd International Athletic Foundation World Symposium on Doping in Sport, Monte Carlo, 1990.

6. Donike M. "Steroid Profiling in Cologne". in Proceedings of the 10th Cologne Workshop on Dope Analysis, Cologne, Germany, 1992. ISBN 3-89001-011-3

Figure 1: T/E ratios from all athletes' samples received during 1990-91.

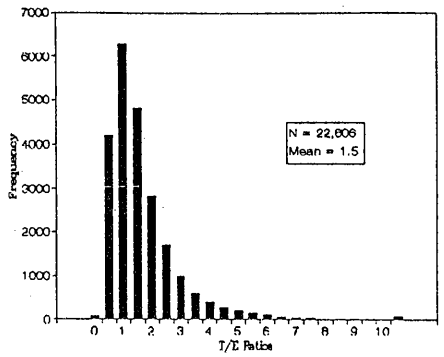


Figure 2: Distribution of T/E ratios from 796 subject athletes (who had 3 or more T/E values) received during 1990-91, and associated with 4,310 individual results.

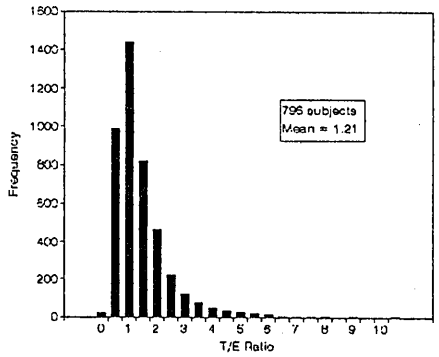


Table 1: Percentile cut-off levels for T/E ratios from the populations of all athletes, subjects from this study, and subsets of study subjects.

Statistic/Percentile Cut-off	#	90%	95%	99%
T/E's of all athletes 1990-91	22806	3-3.5		6.5-7
T/E's of 1285 subjects	4973	2.3	3.2	5.2
T/E Mean's of subjects	1285	2.2	2.9	5.0
T/E Mean's of subjects with 3 or > values	796	2.2	2.9	4.3

USADA1168

Figure 3: Distribution of T/E Means from the 1285 subjects.

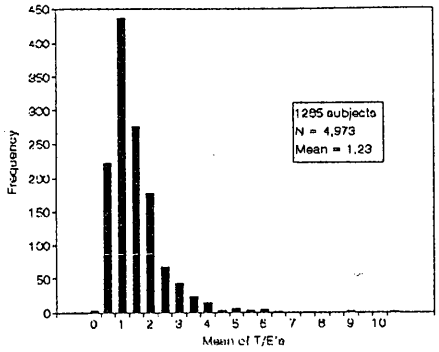


Figure 4: Distribution of T/E Means from those subjects with at least 3 values.

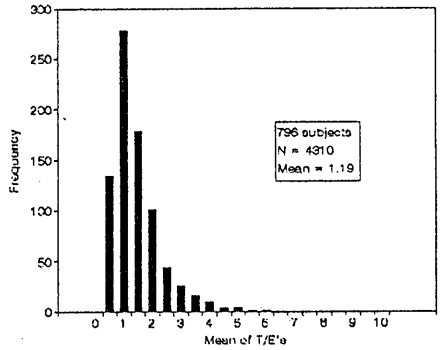


Figure 5: Distribution of the number of T/E values on the 1285 subjects.

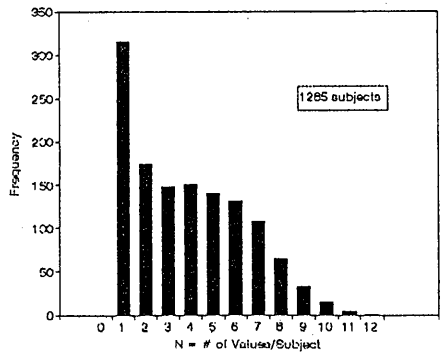


Figure 6: Distribution of subjects' standard deviations of T/E ratios.

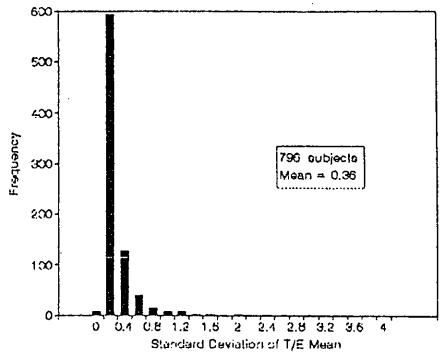


Figure 7: Comparison of subjects' T/E mean versus the standard deviation of the T/E measurements.

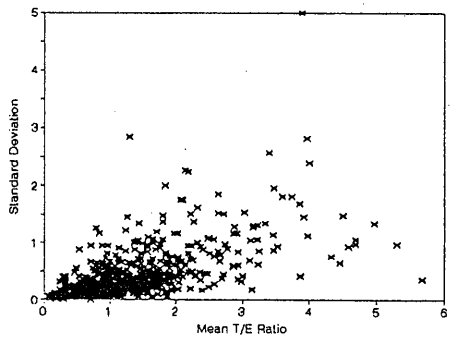


Table 2: Percentile cut-off levels for the standard deviation (SD) determinations grouped by T/E (mean) ranges.

Statistic/Percentile Cut-off	#	90%	95%	99%
SD of T/E's 0-1	405	0.38	0.52	0.95
SD of T/E's 1-2	284	0.81	1.01	1.45
SD of T/E's 2-3	68	1.5	1.76	2.24
SD of T/E's > 3	35	1.96	2.16	2.8
SD of T/E's of all subjects	796	0.87	1.16	1.96

Figure 8: Distribution of Percent Coefficient of Variation of subjects' T/E measurements.

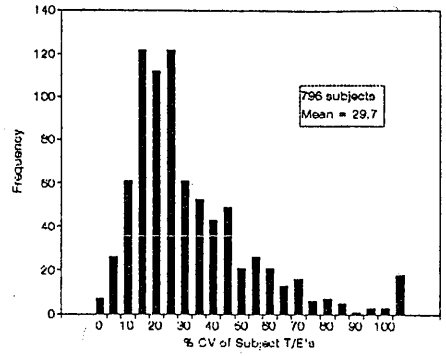


Figure 9: Comparison between the Percent Coefficient of Variation (%CV) of the subjects' T/E measurements versus the subject's mean T/E.

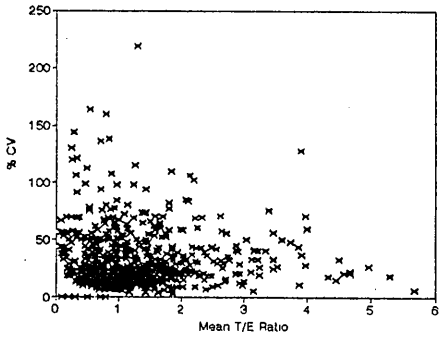


Table 4: Percentile cut-off level for the Percent Coefficient of Variation (%CV) of the subjects' T/E measurements grouped by T/E (mean) ranges.

Statistic/Percentile Cut-off	#	90%	95%	99%
%CV of T/E's 0-1	405	57	75	136
%CV of T/E's 1-2	284	55	68	98
%CV of T/E's 2-3	68	58	70	102
%CV of T/E's > 3	35	56	71	75
%CV of T/E's of all subjects	796	58	72	122

Figure 10: Type 1 pattern of T/E variability (33 of 97 subjects) examples showing low variability; ie %CV < 30, and T/E > 3. (A = 6 %CV; B = 20 %CV; C = 19 %CV)

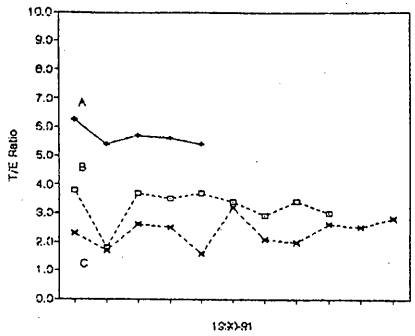


Figure 11: Type 2 pattern of T/E variability (38 of 97 subjects) examples showing a "spike" change in an athlete's T/E ratios.

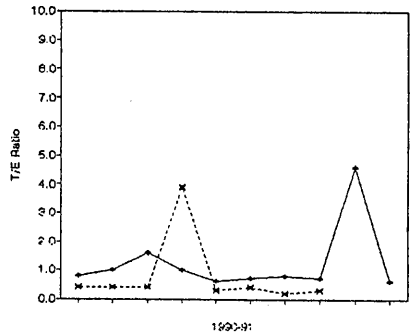
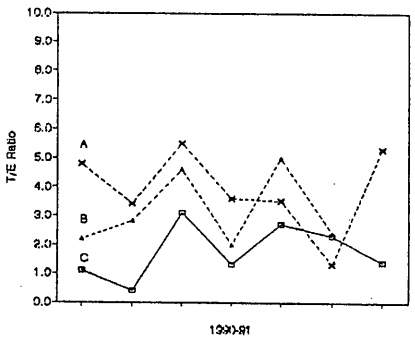


Figure 12: Type 3 pattern of T/E variability (21 of 97 subjects) examples showing a high degree of variability; ie %CV > 30 and T/E above 3. (A = 37 %CV; B = 55 %CV; C = 41 %CV)



USADA1170

Clinical Chemistry 43:7
1280–1288 (1997)

Doping in Sport Symposium

Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athletes' urine

DON H. CATLIN,[1,2]* CAROLINE K. HATTON,[1] and SANJA H. STARCEVIC[1]

Over the last decade the number of laboratories accredited by the International Olympic Committee (IOC) has grown to 25. Nearly half of the ~90 000 samples tested annually are collected on short notice—the most effective means to deter the use of anabolic androgenic steroids (AAS). The major urinary metabolites of AAS have been characterized and are identified by their chromatographic retention times and full or partial mass spectra. The process of determining if an athlete has used testosterone (T) begins with finding a T to epitestosterone (E) ratio >6 and continues with a review of the T/E–time profile. For the user who discontinues taking T, the T/E reverts to baseline (typically ~ 1.0). For the extremely rare athlete with a naturally increased T/E ratio, the T/E remains chronically increased. Short-acting formulations of T transiently increase T/E, and E administration lowers it. Among ancillary tests to help discriminate between naturally increased T/E values and those reflecting T use, the most promising is determination of the carbon isotope ratio.

INDEXING TERMS: sports medicine • epitestosterone

Testing for Anabolic Steroids

ACCREDITED LABORATORIES

In 1983 five laboratories were accredited by the International Olympic Committee (IOC)[3] to perform national and international sport drug testing. Now there are 25 accredited laboratories, and several more are training and preparing for the initial inspection and examination. The accreditation process is initiated by a recommendation from the National Olympic Committee and a commitment from the Committee or other relevant national authority to support the laboratory. The laboratories are reaccredited annually on the basis of performance on one annual examination with blind samples and several proficiency tests. In addition, the laboratories are obliged to adhere to rules and regulations laid down in the IOC Medical Code *[1]*. Of these, the most important is a provision that forbids testing samples from athletes outside of a regulated program that includes sanctions.

For a program to help deter athletes from using drugs, an important factor is geographical access to a laboratory; thus, the ideal distribution of laboratories would be in proportion to population. This is not yet the case, but it is improving. Seventeen of the laboratories are in Europe, three in North America, three in Asia, and one in Australia; South Africa is the most recent addition. There is still no laboratory in South America or the Middle East. A second, equally important factor is the number of tests performed on the national athletes. In some countries, Olympic-caliber athletes are tested many times a year by the national authority and may be tested other times by an International Federation. In other countries, where there is little or no national testing, an elite athlete may be tested only at major national or international competitions.

RESULTS REPORTED

The total number of tests conducted by IOC-accredited laboratories and the percentage of those positive for anabolic steroids are shown in Fig. 1. The total number of tests performed in 1994, the latest year for which data are available, was 93 680, compared with 33 982 in 1986. The graph shows rapid growth in the late 1980s, with a peak annual increase of 36% between 1989 and 1990. This corresponds to a time when sport organizations and the

Departments of [1] Molecular and Medical Pharmacology and [2] Medicine, University of California–Los Angeles, Los Angeles, CA. 90025-6106.
*Address correspondence to this author, at: UCLA Olympic Analytical Laboratory, 2122 Granville Ave., Los Angeles, CA. 90025-6106. Fax 310-206-9077; e-mail dcatlin@UCLA.edu.

[3] Nonstandard abbreviations: AAS, anabolic androgenic steroids; T, testosterone; E, epitestosterone; IOC, International Olympic Committee; OOC, out-of-competition; HRMS, high-resolution mass spectrometry; SIM, selected-ion monitoring; CIR, carbon isotope ratio; LH, luteinizing hormone; TG, TS, T glucuronides and sulfates, respectively; EG, ES, E glucuronides and sulfates, respectively.

Received November 11, 1996; revised March 17, 1996; accepted March 27, 1997.

USADA1171

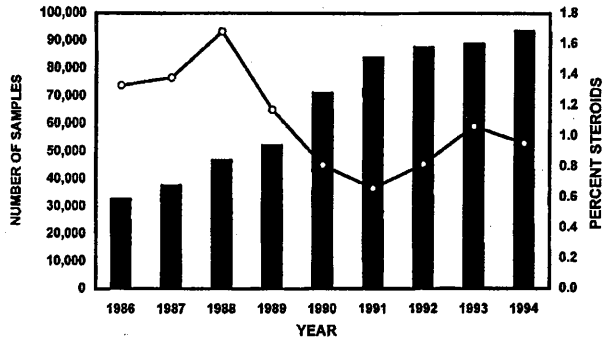


Fig. 1. Total number of urine samples tested in IOC-accredited laboratories by year (*bars*); the *curve* shows the percent of all samples that were reported positive for anabolic steroids.

media were particularly focused on the influence of drugs on sport. In the 1990s, the annual growth has tapered off, averaging 3.5% for the last 3 years shown in Fig. 1. Of greater importance than the number of tests is the proportion of tests performed on samples collected out-of-competition (OOC), i.e., samples collected on short notice or with no notice. OOC testing is the most effective deterrent to the use of anabolic androgenic steroids (AAS). In 1987, the percentage of total tests that was OOC testing was 17%, whereas in 1993 and 1994 this was 39% and 43%, respectively. It is encouraging that the percentage of OOC tests continues to increase at a rate equal to or greater than the total growth rate.

Laboratories that contribute data such as those reported in Fig. 1 are asked to classify the samples into the categories of major international competition, international competition, national competition, and OOC. Ideally, the OOC competition category should be further subdivided by the actual amount of time elapsed between notice to test and collection of the sample—because some steroids have effective half-lives of detection of just a few hours. Some of the OOC samples in Fig. 1 may represent samples collected after as much as 3 days' notice and others after virtually no notice. In some countries, virtually all OOC tests are no-notice tests.

The percentage of samples that test positive for AAS (Fig. 1) provides some information on the trend of usage; however, the lack of further detail precludes conclusions about individual sports or subsets of athletes according to level of performance. For example, an elite athlete who competes several times a year in track and field will probably give many samples, both in-competition and OOC, over the course of a year. Yet Fig. 1 gives only the total number of tests, not the number of athletes tested. Further, the data do not take into consideration the ultimate disposition of the case. For example, not all cases illustrated in Fig. 1 result in sanctions, for various factors that influence the adjudication process *[2]*. Despite the shortcomings in the available data, however, evidence is ample that a growing number of nations and sport federations are conducting credible testing.

Detection of Xenobiotic AAS

In 1994 about two-thirds of the steroid-positive cases shown in Fig. 1 were xenobiotic AAS. For these cases, detection is based on identifying the parent drug or metabolite(s) or both. Identification consists of obtaining the chromatographic retention time or relative retention time and the mass spectrum of the substance and showing that the (relative) retention time and spectrum match that of a reference compound. Fig. 2 shows the total ion chromatogram for an analyzed urine from a male who ingested boldenone. The peaks corresponding to boldenone (17β-hydroxyandrosta-1,4-dien-3-one) and two major metabolites (17β-hydroxy-5β-androst-1-en-3-one and 3α-hydroxy-5β-androst-1-en-17-one) are emphasized *[3]*. The spectra of these three peaks matched those of known (reference) compounds (data not shown). In this case the analysis identified not only a xenobiotic steroid but also two metabolites of the steroid. Thus the amount of analytical information obtained in this analysis is far beyond simple identification of a single substance; indeed, this analysis is helpful in responding to assertions that the sample might have been fortified, because the finding of metabolites is legal evidence that the individual who submitted the urine ingested the substance.

The ability of the laboratories to detect xenobiotic AAS continues to improve, given a growing body of knowledge of the metabolism of the compounds, method improvements, and instrument advances. The major metabolites of virtually all AAS are known and many have been synthesized *[4]*. For some drugs, as many as 11 metabolites have been detected *[5]*. The recent development of methods for analysis by high-resolution mass spectrometry (HRMS) is an important, but expensive, advance. HRMS operates in the selected-ion monitoring (SIM) mode to screen for xenobiotics and in the full-scan mode for confirmations *[6]*. This approach is particularly adept at screening for long-lasting metabolites of stanozolol and methandienone. As in all sports drug testing, a confirmation assay is performed on samples that screen positive. Note that the HRMS-based method described by Schänzer et al. *[6]* uses immunoextraction or HPLC to clean up the

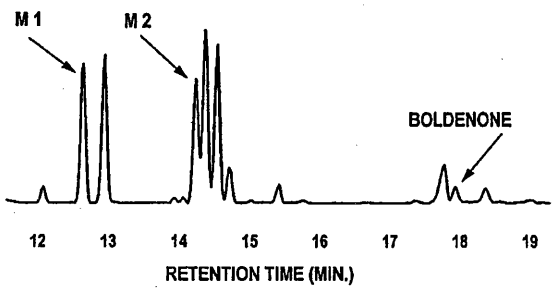


Fig. 2. Total ion current chromatogram of a urine sample collected from a subject who ingested boldenone.

The *arrows* point to boldenone (17β-hydroxyandrosta-1,4-dien-3-one) and two major metabolites: 17β-hydroxy-5β-androst-1-en-3-one (*M1*) and 3α-hydroxy-5β-androst-1-en-17-one (*M2*).

USADA1172

sample extracts before HRMS confirmation of stanozolol or methandienone metabolites, respectively.

One aspect of identification that continues to be debated is the absolute amount and nature of analytical information that must be obtained before a positive report is issued. Some advocate obtaining a full ion scan of at least one of the substances named in the positive report. Others find that SIM is sufficient, and still others use SIM with the major ions bracketed by the adjacent masses. Monitoring the preceding nominal mass allows demonstration that it is not substantially more intense than the ion of interest and thus excludes the possibility that the latter is an isotopic peak from some interference. The subsequent mass must have the expected response. Generally, drug-testing programs in the context of athletics ascribe to the full-scan approach for at least one of the substances reported. This is in contrast to the widely accepted practice in drugs-of-abuse testing of reporting positive cases on the basis of SIM data *[7, 8]*.

Another aspect of xenobiotic steroid testing that merits review is the question of the origin of the steroid and metabolites. One report *[9]* describes the finding of small amounts of boldenone and two metabolites in the urine of a normal man who had not received boldenone. This case argues for caution in interpretation of positive tests for low quantities of boldenone, for additional studies of this issue, and for devising ways to discriminate between endogenous and exogenous sources of boldenone. It is illegal in most countries to treat or feed cattle with anabolic steroids, and European countries in particular vigorously monitor meat for contamination with anabolic steroids; nevertheless, evidence of contamination exists *[10]*. Debruyckere et al. *[11]*, investigating the possibility that contaminated meat could result in steroids or their metabolites in human urine, found that some meat obtained from butcher shops contained clostebol acetate and that its ingestion led to excretion of a clostebol metabolite (4-chloro-delta-4-androstene-3-alpha-ol-17-one *[9]*) in urine. The possibility that urine from untreated subjects may contain very low concentrations of nandrolone metabolites has been discussed *[12, 13]*; however, the mass spectrum of these substances has not been presented, and others find no evidence for nandrolone metabolites in human urine after ingestion of meat from animals that were treated with nandrolone decanoate 28 and 61 days before slaughter *[14]*. Clenbuterol has been reported in very small amounts (<1 ng/L) in some human urines after ingestion of meat from clenbuterol-treated animals *[14]*. In an experiment designed to show human contamination, ingestion of meat from chickens deliberately treated with methenolone heptanoate led to finding methenolone and metabolites in urine *[15]*.

Detection of Doping with Testosterone (T)

Doping with endogenous steroids is the most serious issue facing sport today; when cleverly administered, these are very difficult to detect. Quadrupole mass spectrometers cannot distinguish between pharmaceutical T and natural T because their spectra are identical. The average male produces 6–10 mg of T per day, of which only ~1% is excreted in urine. The urine concentration of T increases briefly after T administration; however, T has a short half-life (~1 h) and its concentrations fall rapidly *[16]*. Even T enanthate, an ester that increases concentrations of T in serum for ~2 weeks, often does not cause an obvious increase in urine T. Urinary androsterone and etiocholanolone account for ~70% of a dose of T *[17]*; however, many other endogenous steroids are metabolized to androsterone and etiocholanolone, and neither their concentrations nor their ratio is a useful marker of T administration. The ratio of androsterone to epitestosterone (E; 17α-hydroxy-androst-4-en-3-one) has some utility as a marker of recent T use.

Various approaches to prove T administration have been considered within the practical constraint that only one untimed urine is available for the screening test. Brooks et al. *[18]* pointed out that urine concentrations vary over a very wide range such that defining T doping as a concentration that exceeds an upper limit of normal would be difficult. Instead, they advocated measuring the ratio of T to luteinizing hormone (LH) in urine, because chronic administration of T inhibits the production of LH and lowers the urine concentration of LH. Detailed studies of urine T/LH ratios are available *[19]*. Because the T/LH and other urine ratios are independent of urine volume and other factors that influence concentration, such ratios have been used extensively to detect doping with endogenous substances. One major drawback of T/LH is the lack of a reference method for LH *[20]*.

Doping with T was reported in the 1950s *[21]*, but not until 1982 did an effective test became available: Donike et al. *[22]* proposed to detect T doping by monitoring the T/E ratio. E is the 17α epimer of T and is present in urine in concentrations similar to T. T and E differ chemically only in the configuration of the hydroxyl group on C-17. T is not metabolized to E *[23]*, and T/E increases after T administration. Subsequently, Dehennin and Matsumoto pointed out that T administration lowers the concentration of E *[24]*; thus, the increase in T/E results from an increase in T and a decrease in E.

In 1982 the IOC Medical Commission defined a T/E >6:1 as the dividing line between the presumed upper limit of normal T/E values and doping with T. Since then, a small number of cases have been found with T/E >6:1 but without the subjects having been administered T *[25–28]*. Therefore, the definition of T doping has been changed: Individuals with T/E >6:1 undergo further testing before a conclusion is reached on the etiology of the increased T/E. The shape of the distribution of T/E values for 3710 male athletes (Fig. 3) is skewed, having a median of 1.1, and is 5.6 or less in 99% of the athletes tested. Of those with T/E >6:1, some admitted using T and others denied ever using it. Moreover, a T/E that is not >6 does not mean that the individual has not used T

USADA1173

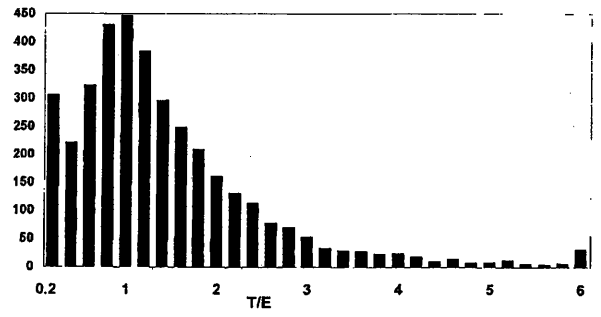


Fig. 3. Distribution of urine T/E in 3710 urines collected from male football players participating in a steroid detection program.

In most cases the athletes had <48 h notice of the test. The upper end of the distribution is such that the highest bin contains all T/E values >6:1. The highest three ratios were 30, 36, and 57. *Ordinate*, no. of samples.

recently; rather, it is consistent with recent use of short-acting T, use of T and E together, use of E to camouflage recent T use, or no use of T. When T/E is not >6, the laboratory issues a negative report, recognizing that it may be a describing a false-negative sample.

T/E–TIME PROFILE

In view of the many interpretations of an increased T/E value in a single urine, a strategy is needed for managing individual cases. If the athlete has never been tested before, one should try to obtain at least two additional samples at 3- to 6-week intervals, with as short a notice as possible. Next, plot the T/E results against time and calculate the mean, SD, and CV. Stable T/E values are interpreted as consistent with physiological or natural increases of T/E, and decreasing T/E values indicate that the high result reflected T administration. If the individual has been tested two or more times before the increased T/E result is obtained, and these T/E values are near or less than the median T/E for males (~1.0), then the high T/E is a strong indication of T administration. The actual value of the T/E is very helpful in differentiating natural increases from T administration, particularly if the T/E is 15 or higher. At those values, the likelihood of natural increases is remote. Conversely, the closer the high T/E is to 6:1, the more likely is the possibility of a physiological etiology.

Our data on urinary T/E ratios from drug-free males reveals that, for three or more samples taken at monthly or greater intervals, the CV will be <60%; 55% is the maximum we have observed. Fig. 4 shows the T/E–time profiles for three athletes. Athletes B and C have a mean T/E of 1.0 and 2.3 and CVs of 9.8% and 39.5%, respectively. Athlete A, whose initial T/E was 8.0, has a mean of 2.7 and CV of 105%; his pattern is typical of a T user who gets caught and discontinues T. In our experience, most T users who give three or more samples will have a CV >60%. This is in agreement with the data of Garle et al. *[29]* and appears to be consistent with the athletes who displayed a "T/E spike" pattern described by Baenziger and Bowers *[30]*.

Many males will have a remarkably stable T/E over several months or years *[31–33]*. For example, the volunteer in Fig. 5 has a stable T/E over the first 9 consecutive days (CV 3.5%) and in intermittent assays over 8 months (CV 35%). Donike et al. reported *[32]* that the CV (in normal males) will not exceed 30%; however, in a study of 796 athletes who provided at least 3 urines over 2 years, Baenziger and Bowers *[30]* showed (their Figs. 8 and 9) that about one-third had CVs >30%, and that the 90th percentile for the CV of T/E ratios was 58%. Further, in a study *[29]* of 28 athletes with at least one T/E >6, 17 (61%) had CVs <30%, but 10 (36%) had CVs of 31–43%; the authors regarded the former group as "not likely to be testosterone users," and the latter as being in a "grey area." In the only study that covers several months of sampling in females (2 samples a month for 12 months, 5 subjects), Mareck-Engelke et al. *[33]* reported CVs of 15%, 51%, 25%, 31%, and 30%. Thus, although CVs are a useful guideline to understanding the T/E–time profile of an athlete, various factors may influence the T/E (see below, and *[34]*); therefore, the interpretation of a profile that includes one sample with T/E >6 should take into account all the available information for the subject tested.

The role of quantification of concentrations of T and E is important in the interpretation of T/E; however, the most useful information is the overall pattern of the T/E–time profile. Analytical and instrumental factors that influence the determination of T/E need to be taken into consideration *[35, 37]*. If the actual T/E value is reported, it is important to have documentation of the confidence interval of the measurement, in case that information is requested. Likewise, if the sample is reported as >6:1, it is important to have documentation of the statistical criteria used to make the decision.

The precise value of T/E is less critical if the value is two or more times the 6:1 threshold for reporting. The rationale for caution is that precise numbers are readily attacked by litigation attorneys, and lay-adjudicators often do not understand measurement variability. For example, the T/E on confirmation A will not be identical to the confirmation B value for T/E, and both will differ from the T/E reported in the screening assay.

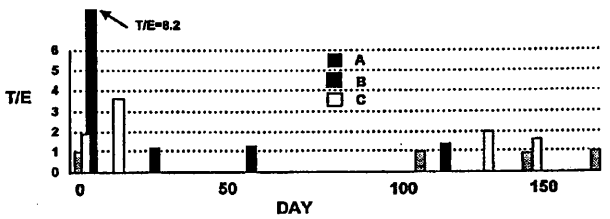


Fig. 4. T/E profiles of three athletes sampled four times over 170 days on relatively short notice.

The T/E of the first sample from athlete A was 8.2, followed by subsequent T/Es of 1.2, 1.3, and 1.4 (mean = 3.0, CV = 114%). The T/Es from athlete B showed minimal variability (mean = 0.97, CV = 9.8%); athlete C revealed greater variability (mean = 2.3, CV = 40%) but gave ratios still within our norms for non-T-using controls.

USADA1174

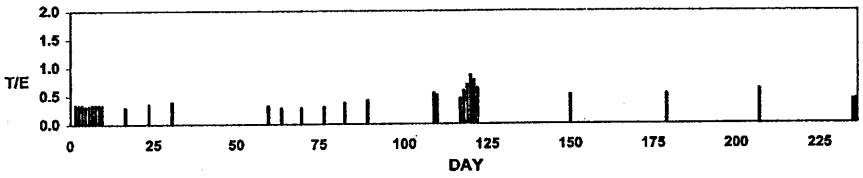


Fig. 5. T/E ratios of a drug-free volunteer who contributed 31 samples over 8 months.

For the first 9 days a sample was collected each day, all were analyzed in one batch, and the CV was only 3.5%. For the second period of consecutive daily samples shown, the CV was 21%. The CV for all samples was 35%.

In many cases of increased T/E, the concentration of E is low (<5 μg/L). Because the CV of the assay increases at low concentrations of T or E (or both), the corresponding CV of the T/E may be relatively high. Low concentrations of T are not an issue with T/E ratios; however, very high concentrations of T (>300 μg/L) are an indication of recent T administration. Expressing T and E concentrations in nanograms per milligram of creatinine is useful, but this approach has not been widely used, probably because of concerns that creatinine excretion rates are influenced by exercise and many other factors *[26]*.

INCREASED T/E

T/E between 6:1 and 10:1. If an athlete produces three or more urine samples with T/E >6:1, none with T/E <6:1, and a CV <60%, we tentatively classify the results as naturally increased and recommend additional tests. We also take into consideration the absolute value of the E and the amount of advance notice for the collections. If, at this point, a clinical examination and serum tests have not been performed, we recommend serum tests that are routinely available to the clinician: complete blood count; routine chemistries, including HDL and LDL cholesterol; free and total T, sex-hormone-binding globulin, dehydrotestosterone, follicle-stimulating hormone, and LH; urine LH; and a complete medical history and physical examination, with emphasis on the hypothalamic–pituitary–testicular axis and the adrenal. If these evaluations are normal, the likelihood of a T-secreting tumor is extremely remote. If the serum T is increased or if the follicle-stimulating hormone or LH is low, or both, it is important to extend the evaluation and determine if there is an endogenous source of T or the use of exogenous T. In difficult cases, a 24-h urine may be collected; analyzed for the total amount of T, androsterone, and etiocholanolone; and the results compared with published norms *[39]*. For a few of the athletes we monitor, serum tests and clinical evaluation have been performed and the results have been normal.

T/E >10:1. In this situation, we still recommend a minimum of two additional tests, even though we have never encountered a case of potential physiologically increased T/E where the mean is >15:1. If the first sample tested from an athlete exceeds 15:1, we expect the subsequent T/E values to decrease to <6:1; in all cases where three additional samples have been obtained, T/E did fall to <6:1. If the index T/E is 10–15:1, a later T/E will decline to <6:1 in most, but not all, cases. When it does not, we recommend the clinical evaluation described above and, if possible, one or more of the ancillary tests described below.

Recently, we have encountered several cases where the T/E has remained in the 9–13:1 zone despite as many as five additional samples collected with <24-h notice (Fig. 6). These cases are a growing concern because they raise the specter of sophisticated T delivery systems that can produce stable yet very high T/E values; moreover, they may be increasing in frequency.

ANCILLARY TESTS TO DETECT T ADMINISTRATION

Invasive tests. In case the above approach still does not lead to a definitive determination of whether or not T has been used, additional tests may be performed—with the understanding that documented experience with these tests is limited and that more-invasive techniques are involved.

The ketoconazole challenge test *[26, 27, 40]* involves collecting urines before and after administration of an oral dose of ketoconazole, which inhibits the synthesis of T. In a normal male, after administration of ketoconazole, urinary T declines, E is unchanged, and T/E decreases *[27, 40]*. Conversely, if the T/E is increased because of T administration, the T/E after ketoconazole administration increases. In individuals who probably have a naturally higher T/E, the ketoconazole test reveals a pattern similar to that of the normal male *[27, 40]*. Experience to date with the ketoconazole test indicates that it differentiates between T users and nonusers. Its greatest potential application is to determine whether individuals suspected of having physiologically increased T/E respond as expected. At this time, the test is not widely used in the US because it requires drug administration and entails the risks related thereto, as well as commitment and expense

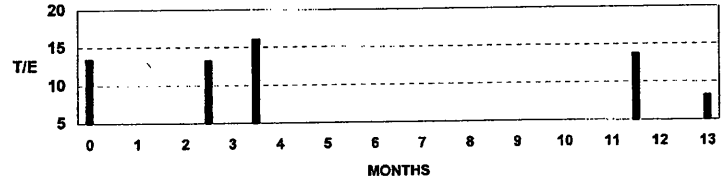


Fig. 6. T/E–time profile for one athlete who gave five samples on short notice over 13 months.

Although the mean of 13 is high, the CV is relatively low (22%).

USADA1175

on the part of the athlete or the sports organization. There is also a sense that athletes with naturally increased T/E should not be coerced into the test for the sake of proving their innocence.

Another approach is based on measuring the concentration in serum of any substance that precedes T in the biosynthetic pathway to T and calculating a ratio of that substance to T; in the presence of exogenous T, the production of the precursors will be suppressed and the ratio should be high. Carlström et al. *[20]* described the serum 17α-hydroxyprogesterone/T ratio, presented the distribution of values for 12 males who did not use T, and showed that the ratio is increased after T administration; in the one subject with chronically increased T/E, the 17α-hydroxyprogesterone/T ratio was within the normal range. More experience with this test is desired.

Noninvasive tests. In typical T/E analysis, the glucuronides and sulfates in the urine samples are hydrolyzed with β-glucuronidase from *Escherichia coli* or *Helix pomatia*. In the former case, the test measures the ratio of total T (unconjugated T plus the T deconjugated from the glucuronide) to total E. In the latter case, the unconjugated glucuronide and to some extent the sulfate fractions are taken into account. Dehennin *[41]* has taken the additional step of measuring the concentrations of T and E glucuronides (TG, EG) and sulfates (TS, ES) plus other precursors of T and E. He used β-glucuronidase to obtain the glucuronide fraction and DEAE-Sephadex to separate the sulfates from the glucuronides and the nonconjugated steroids. He has proposed *[41, 42]* that individuals with T/E >4:1 have a defect in E production, which results in decreased concentrations of EG and normal or increased concentrations of ES. Further, he finds that individuals with T/E >4:1 have low EG/ES ratios, and their TG/(EG+ES) values are less than the increase threshold (mean + 4.5 SD) of the reference group. Additional studies of these and other proposals *[30]* of Dehennin are underway. To that end, Sanaullah and Bowers *[43]* have synthesized deuterium-labeled T and E glucuronides and sulfates and have developed a liquid chromatography–tandem mass spectrometry method for directly quantifying these moieties.

Another noninvasive test is the determination of the carbon isotope ratio (CIR). Most carbon atoms are ^{12}C, and a very few are ^{13}C and ^{14}C. Becchi et al. *[44]* explored the hypothesis that the $^{13}C/^{12}C$ of synthetic T differs from the $^{13}C/^{12}C$ in endogenous T. Using a specialized GC-MS that measures this CIR, they found support for the hypothesis by showing that, after T administration, the $\delta^{13}C$ $^{0}/_{00}$ values for T were <-27, whereas samples from normal controls had values that were less negative than -27. In a subsequent study, the $\delta^{13}C$ $^{0}/_{00}$ values for T, cholesterol, and metabolites of T were determined for 25 samples from 8 apparently healthy volunteers before and after T administration *[45]*. The $\delta^{13}C$ $^{0}/_{00}$ values of T and metabolites were lower after T administration, and discriminant analysis correctly identified the samples collected after T administration *[45]*. More recently, we have measured the $\delta^{13}C$ $^{0}/_{00}$ values of T in 14 urines from three individuals whom we expected to have physiological increases of T/E. The $\delta^{13}C$ $^{0}/_{00}$ values of T in all 14 was between -24 and -27. At this juncture, the CIR studies consistently show that after T administration the $\delta^{13}C$ $^{0}/_{00}$ values of T are -30 to -36 if the T/E is >10; however, the studies to this point have not included enough samples from T users with T/E ratios in the critical range of 4:1 to 10:1, so the sensitivity of the CIR test is not known. Although it was encouraging to find high T $\delta^{13}C$ $^{0}/_{00}$ values in three subjects presumed to have physiologically increased T/E, more data are needed. Further on-going studies are anticipated to improve on the measurement of CIR and on the premeasurement analytical techniques.

OTHER VARIABLES INFLUENCING T/E

Ethanol. In 1992 Falk et al. *[46]* measured the T/E before and after administering ethanol (2 g/kg) over 4 h to four males. The ratio increased by 60% in one, ~20% in two, and no change in the fourth *[46]*. Subsequently, papers presented at a workshop on doping *[47–49]* found differing effects of ethanol on the T/E. Males showed no significant effect of ethanol on T/E at doses of 1.0–1.2 g/kg *[46, 49]*, whereas the same dose produced large increases in another study *[36]*. At very high doses of ethanol (2 g/kg), the T/E ratios of males increased, although none exceeded 6:1 *[46, 47, 49]*. The T/E ratios in females appear to be more sensitive to ethanol. Doses of 1.0–1.2 g/kg produced either no effect on T/E *[49]* or increases *[48]*, and high doses produced increases *[47, 49]*, including two that exceeded 6:1 *[48, 49]*. The effect of ethanol on T/E appears to be limited to the 8 h after completing the ingestion. It will be important to advance these studies by adding placebo controls to factor out the effects of biorhythms and other sources of potential bias and to clarify dose–response relationships. Until further details are available, one may prudently consider that large and inebriating doses of ethanol may increase the T/E for several hours after ingestion and that some females may be particularly sensitive to the ethanol effect.

Gender. Little is published about gender differences in T/E; however, in our experience, the T/E distribution of a control group of female students, who are not at risk to take T, is shifted slightly to the left (lower values) of the distribution for control males. The reason for this is not known. We have found, however, that the menstrual cycle may influence T/E. We obtained daily T/E values on morning urines from three females throughout a total of five menstrual cycles; each showed a peak during menses, and the nadir-to-peak T/E ratio could vary by nearly threefold over the course of a cycle. Data on three cycles in one of these subjects have been presented *[50]*. In other studies that collected 24-h urines from four females on days 2, 7, 14, and 21 of the menstrual cycle *[33]*, at the

USADA1176

beginning and at mid-cycle for 1 year (n = 5) *[51]*, there was no evidence of a relationship between T/E and the menstrual cycle. Additional studies using identical protocols will help clarify the relation between T/E and the menstrual cycle.

Masking Testosterone Administration

Short half-life formulations of T. Currently, the drug of choice for the management of hypogonadal syndromes is T enanthate, typically given by injection every 2 or 3 weeks in doses of 100–200 mg. Because parenteral injections are inconvenient, the pharmaceutical industry has been developing alternative formulations and routes of administration. T cyclodextrin is a formulation administered by having the patient place a tablet in the buccal-sublingual pouch to be absorbed into the bloodstream. A dose of 5 mg produces serum concentrations of T within the reference range for normal *[52]* but, because of the very short half-life of T, the drug must be taken three times per day. This preparation leads to very high urinary ratios of T/E, but these typically fall to <6:1 within 4–6 h (Fig. 7) *[55]*. Two T formulations that utilize the transdermal route of administration became available recently and others are in development *[53, 54]*. One of these is a T gel that is administered by applying the gel on the skin. As expected, this formulation also increases the urine T/E (Fig. 7). (These studies were approved by the institutional review board.) The question remains whether or not these preparations can be administered in sufficient doses to enhance performance. The threshold dose for a pharmacological effect of T in men is not known, but 600 mg/week of T enanthate for 10 weeks is known to increase fat-free body mass and muscle size and strength in healthy men *[56]*. Given that T cyclodextrin is readily bioavailable, we can reasonably suspect that it would provide enhancement.

Administration of E. Anecdotal reports suggest that E is used as an emergency measure to rapidly lower a T/E that is above-normal as a result of T administration. E is not available in a pharmaceutical dosage form but it is available as a chemical. For this reason the IOC Medical Commission classified E as a urine-manipulating agent, set 150 μg/L (520 nmol/L) as the threshold for reporting cases, and later changed the threshold to 200 μg/L (693 nmol/L). In our opinion the threshold should be higher: We often encounter cases in the 150–200 μg/L range. In the future we expect that the CIR technique might be useful for detecting E administration. The two highest E concentrations we have encountered in urine samples were 1200 (4.16 μmol/L) and 1550 μg/L (5.20 μmol/L).

Perhaps the ideal doping agent would be a combination of T and E designed to deliver T and E in a ratio of ~25:1, i.e., the ratio of production rates of T and E in men *[42]*. In theory, this might produce a T/E of 1:1 and therefore produce false-negative test results while allowing administration of T. In practice, the dosing regimens do not always lead to T/E <6:1, and the formulations are cumbersome to prepare. Moreover, Dehennin *[42]* proposes to detect this scheme by measuring the ratio of T and E to 5-androstene-3β,17α-diol, a precursor of E, and Kicman et al. *[19]* have shown that the T/LH ratio is high after administration of combined T and E.

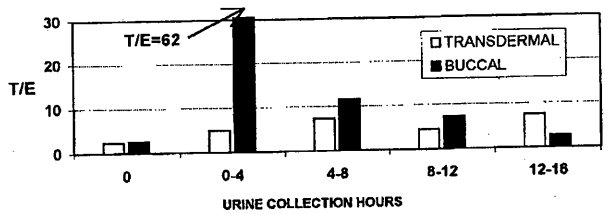


Fig. 7. T/E ratios in urines collected at 4-h intervals after transdermal and sublingual-buccal administration of T to two subjects.

The sublingual formulation contained 25 mg of T embedded in cyclodextrin. The transdermal formulation contained 100 mg of T.

We thank the National Collegiate Athletic Association, the US Olympic Committee, and the National Football League for providing research support that enabled the studies referred to herein. We thank C. Wang and R. S. Swerdloff of Harbor–University of California–Los Angeles Medical Center for collaboration on the testosterone cyclodextrin and transdermal testosterone study, and their sources of support (Biotechnology General Corp., Iselin, NJ, and the Harbor–UCLA Clinical Research Center).

References

1. IOC. Medical code. Lausanne, Switzerland: International Olympic Committee, 1995; ISBN 92-9149-003-2.
2. Catlin DH, Murray TH. Performance-enhancing drugs, fair competition, and Olympic sport. JAMA 1996;276:231–7.
3. Schänzer W, Donike M. Metabolism of boldenone in man: gas chromatographic/mass spectrometric identification of urinary excreted metabolites and determination of excretion rates. Biol Mass Spectrom 1992;21:3–16.
4. Schänzer W. Metabolism of anabolic androgenic steroids [Review]. Clin Chem 1996;42:1001–20.
5. Schänzer W, Opfermann G, Donike M. Metabolism of stanozolol: identification and synthesis of urinary metabolites. J Steroid Biochem 1990;36:153–74.
6. Schänzer W, Delahaut P, Geyer H, Machnik M, Horning S. Long term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography–high-resolution mass spectrometry. J Chromatogr B 1996;687:93–108.
7. Critical issues in urinalysis of abused substances: report of the substance-abuse testing committee. Clin Chem 1988;34:605–32.
8. National Laboratory Certification Program. Guidance document for laboratories and inspectors. Washington, DC: Department of Health and Human Services, Substance Abuse and Mental Health Services Administration, 1994:123–4.
9. Schänzer W, Geyer H, Gotzmann A, Horning S, Mareck-Engelke U, Nitschke E, et al. Endogenous production and excretion of boldenone (17β-hydroxyandrosta-1,4-dien-3-one), an androgenic ana-

USADA1177

bolic steroid. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 12th Cologne workshop on dope analysis, 10–15 April 1994. Edition Sport, Köln: Sport und Buch Strauss, 1995:199–211.

10. Daeseleire EAI, Guesquiére AD, Van Peteghem CH. Multiresidue analysis of anabolic agents in muscle tissues and urine of cattle by GC-MS. J Chromatogr Sci 1992;30:409–14.
11. Debruyckere G, de Sagher R, Van Peteghem C. Clostebol-positive urine after consumption of contaminated meat. Clin Chem 1992; 38:1869–73.
12. Bjorkhem I, Ek H. Detection and quantitation of 19-norandrosterone in urine by isotope dilution–mass spectrometry. J Steroid Biochem 1982;17:447–51.
13. Debruyckere G, Van Peteghem CH. Influence of the consumption of meat contaminated with anabolic steroids on doping tests. Anal Chim Acta 1993;275:49–56.
14. Hemmersbach P, Tomten S, Nilsson S, Oftebro H, Havrevoll O, Oen B, Birkeland K. Illegal use of anabolic agents in animal fattening—consequences for doping control. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 12th Cologne workshop on dope analysis, 10–15 April 1994. Edition Sport, Köln: Sport und Buch Strauss, 1995:185–92.
15. Kicman AT, Cowan DA, Myhre L, Nilsson S, Tomten S, Oftebro H. Effect on sports drug tests of ingesting meat from steroid (methenolone)-treated livestock. Clin Chem 1994;40:2084–7.
16. Sandberg AA, Slaunwhite WR. Metabolism of 4-^{14}C-testosterone in human subjects. I. Distribution in bile, blood, feces and urine. J Clin Invest 1956;35:1331–9.
17. Deslypere JP, Wiers PW, Sayed A, Vermeulen A. Urinary excretion of androgen metabolites, comparison with excretion of radioactive metabolites after injection of [4-^{14}C]testosterone: influence of age. Acta Endocrinol (Copenh) 1981;96:265–72.
18. Brooks RV, Jeremiah G, Webb WA, Wheeler M. Detection of anabolic steroid administration to athletes. J Steroid Biochem 1979;11:913–7.
19. Kicman AT, Brooks RV, Collyer SC, Cowan DA, Nanjee MN, Southan GJ, et al. Criteria to indicate testosterone administration. Br J Sports Med 1990;24:253–64.
20. Carlström K, Palonek E, Garle M, Oftebro H, Stanghelle J, Björkhem I. Detection of testosterone administration by increased ratio between serum concentrations of testosterone and 17α-hydroxyprogesterone. Clin Chem 1992;38:1779–84.
21. Ziegler JB. Introduction. In: Goldman B, ed. Death in the locker room. South Bend, IN: Icarus Press, 1984:1–3.
22. Donike M, Barwald KR, Klostermann K, Schänzer W, Zimmermann J. Nachweis von exogenem Testosteron [Detection of exogenous testosterone]. In: Heck H, Hollmann W, Liesen H, et al., eds. Sport: Leistung und Gesundheit, Kongressbd. Dtsch Sportarztekongress. Köln: Deutscher Ärzte-Verlag, 1983:293–8.
23. Korenman SG, Wilson H, Lipsett MB. Isolation of 17-alpha-hydroxyandrost-4-en-3-one (epitestosterone) from human urine. J Biol Chem 1964;239:1004–6.
24. Dehennin L, Matsumoto AM. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. J Steroid Biochem Mol Biol 1993;44: 179–89.
25. Namba O, Miyachi Y, Kawahara M, Kuroda I, Kuroda Y. Urinary testosterone and epitestosterone secretion in a doping positive subject. In: Laron Z, Rogol AD, eds. Hormones and sport. New York: Raven Press, Serono Symposia Publications, 1989:275–83.
26. Oftebro H. Evaluating an abnormal urinary steroid profile. Lancet 1992;339:941–2.
27. Kicman AT, Oftebro H, Walker C, Norman N, Cowan DA. Potential use of ketoconazole in a dynamic endocrine test to differentiate between biological outliers and testosterone use by athletes. Clin Chem 1993;39:1798–803.
28. Raynaud E, Audran M, Brun JF, Fedou C, Chanal JL, Orsetti A. False-positive cases in detection of testosterone doping. Lancet 1992;340:1468–9.
29. Garle M, Ocka R, Palonek E, Björkhem I. Increased urinary testosterone/epitestosterone ratios found in Swedish athletes in connection with a national control program evaluation of 28 cases. J Chromatogr B 1996;687:55–9.
30. Baenziger J, Bowers L. Variability of T/E ratios in athletes. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 11th Cologne workshop on dope analysis, 7–12 March 1993. Edition Sport, Köln: Sport und Buch Strauss, 1994, 41–52.
31. Catlin DH. Androgen abuse by athletes. In: Bhasin S, Gabelnick HL, Spieler JM, Swerdloff RS, Wang C, Kelly C, eds. International Androgen Workshop (2nd): pharmacology, biology, and clinical applications of androgens: current status and future prospects. New York: Wiley-Liss, 1995:289–300.
32. Donike M, Rauth S, Mareck-Engelke U, Geyer H, Nitschke R. Evaluation of longitudinal studies, the determination of subject based reference ranges of the testosterone/epitestosterone ratio. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 11th Cologne workshop on dope analysis, 7–12 March 1993. Edition Sport, Köln: Sport und Buch Strauss, 1994:33–9.
33. Mareck-Engelke U, Flenker U, Donike M. Stability of steroid profiles (5): the annual rhythm of urinary ratios and excretion rates of endogenous steroids in female and its menstrual dependency. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 13th Cologne workshop on dope analysis, 12–17 April 1995. Edition Sport, Köln: Sport und Buch Strauss, 1996:177–90.
34. Geyer H, Schänzer W, Mareck-Engelke U, Donike M. Factors influencing the steroid profile. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 13th Cologne workshop on dope analysis, 12–17 April 1995. Edition Sport, Köln: Sport und Buch Strauss, 1996:95–114.
35. Linnet K. Effect of the biological matrix on the urinary testosterone/epitestosterone ratio measured by gas chromatography/mass spectrometry in doping analysis. Biol Mass Spectrom 1993;22:412–8.
36. Catlin DH, Cowan DA, de la Torre R, Donike M, Fraisse D, Oftebro H, et al. Urinary testosterone (T) to epitestosterone (E) ratios by GC/MS. I. Initial comparison of uncorrected T/E in six international laboratories. J Mass Spectrom 1996;31:397–402.
37. Nolteernsting E, Geyer H, Mareck-Engelke U, Schänzer W, Donike M. Standardization of the T/E-determination by deuterated internal standards. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 12th Cologne workshop on dope analysis, 10–15 April 1994. Edition Sport, Köln: Sport und Buch Strauss, 1995:113–20.
38. Boeniger MF, Lowry LK, Rosenberg J. Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review. Am Ind Hyg Assoc J 1993;54: 615–27.
39. Weykamp CW, Penders TJ, Schmidt NA, Borburgh AJ, van de Calseyde JF, Wolthers BJ. Steroid profile for urine: reference values. Clin Chem 1989;35:2281–4.
40. Oftebro H, Jensen J, Mowinckel P, Norli HR. Establishing a ketoconazole suppression test for verifying testosterone administration in the doping control of athletes. J Clin Endocrinol Metab 1994;78:973–7.
41. Dehennin L. On the origin of physiologically high ratios of urinary testosterone to epitestosterone: consequences for reliable detection of testosterone administration by male athletes. J Endocrinol 1994;142:353–60.

USADA1178

42. Dehennin L. Detection of simultaneous self-administration of testosterone and epitestosterone in healthy men. Clin Chem 1994;40:106–9.
43. Sanaullah, Bowers LD. Facile synthesis of [16,16,17-$^2H_3$]-testosterone, -epitestosterone and their glucuronides and sulfates. J Steroid Biochem Mol Biol 1996;58:225–34.
44. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. Rapid Commun Mass Spectrom 1994;8: 304–8.
45. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, et al. Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. J Mass Spectrom 1996;31:169–76.
46. Falk O, Palonek E, Björkhem I. Effect of ethanol on the ratio between testosterone and epitestosterone in urine. Clin Chem 1988;34:1462–4.
47. Mareck-Engelke U, Geyer H, Schindler U, Flenker U, Iffland R, Donike M. Influence of ethanol on steroid profile parameters. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 13th Cologne workshop on dope analysis. Köln: Sport und Buch Strauss, 1995:144–8.
48. Seppenwoolde-Waasdorp TJA, de Boar D, Van Engelen HMJ, Vrijday AD, Maes RAAA. Evaluation of endogenous steroid profiles in urine. 2. Effects of ethanol intake revisited. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 13th Cologne workshop on dope analysis. Köln: Sport und Buch Strauss, 1995:157–65.
49. Leinonen A, Karila T, Seppälä T. An increased testosterone to epitestosterone ratio due to high doses of ethanol—a case report on a female powerlifter. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 13th Cologne workshop on dope analysis. Köln: Sport und Buch Strauss, 1995:167–76.
50. Hatton CK, Catlin DH, Straus PW, Starcevic SH. Urinary steroid profiles: a longitudinal study. Proc., 38th ASMS Conf. on Mass Spectrometry and Allied Topics, Tucson, AZ, June 3–8, 1990: 1004–5.
51. Mareck-Engelke U, Geyer H, Donike M. Stability of steroid profiles (4): the circadian rhythm of urinary ratios and excretion rates of endogenous steroids in female and its menstrual dependency. In: Donike M, Geyer H, Gotzmann A, et al., eds. Proc., 12th Cologne workshop on dope analysis, 10–15 April 1994. Edition Sport, Köln: Sport und Buch Strauss, 1995:135–55.
52. Salehian B, Wang C, Alexander G, Davidson T, McDonald V, Berman N, et al. Pharmacokinetics, bioefficacy, and safety of sublingual testosterone cyclodextrin in hypogonadal men: comparison to testosterone enanthate—a clinical research center study. J Clin Endocrinol Metab 1995;80:3567–75.
53. Cunningham GR, Cordero E, Thornby JI. Testosterone replacement with transdermal therapeutic systems. Physiological serum testosterone and elevated dihydrotestosterone levels. JAMA 1989; 261:2525–30.
54. Meikle AW, Arver S, Dobs AS, Sanders SW, Rajaram L, Mazer NA. Pharmacokinetics and metabolism of a permeation-enhanced testosterone transdermal system in hypogonadal men: influence of application site—a clinical research center study. J Clin Endocrinol Metab 1996;81:1832–40.
55. Catlin DH, Salehian B, Boghosian T, Swerdloff RS, Wang C. Changes in urinary testosterone and epitestosterone after sublingual testosterone cyclodextrin administration [Abstract]. Clin Res 1993;42:74A.
56. Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, et al. The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. N Engl J Med 1996;335: 1–7.

J. Steroid Biochem. Molec. Biol. Vol. 44, No. 2, pp. 179–189, 1993
Printed in Great Britain

0960-0760/93 $6.00 + 0.00
Pergamon Press Ltd

LONG-TERM ADMINISTRATION OF TESTOSTERONE ENANTHATE TO NORMAL MEN: ALTERATIONS OF THE URINARY PROFILE OF ANDROGEN METABOLITES POTENTIALLY USEFUL FOR DETECTION OF TESTOSTERONE MISUSE IN SPORT

LOUIS DEHENNIN[1]* and ALVIN M. MATSUMOTO[2]

[1]Fondation de Recherche en Hormonologie, 67 Boulevard Pasteur, 94260 Fresnes, France and [2]Gerontology Research, Education and Clinical Center, Department of Veterans Affairs Medical Center and Population Center for Research in Reproduction, Department of Medicine, University of Washington, Seattle, WA 98108, U.S.A.

(Received 5 August 1992; accepted 30 September 1992)

Summary—Doses equivalent to 18, 72 and 216 mg testosterone (T)/week were administered for 6 months to eugonadal men. Urinary excretions of androgen glucuronides (G) were quantitatively analyzed by gas chromatography-mass spectrometry with stable isotope dilution during periods of control (without hormone treatment), T administration and recovery. The lowest dosage T administration did not affect the androgen profile, while higher dosages generally increased urinary excretions of T metabolites (TG, T sulfate, glucuronides of androsterone, etiocholanolone, 5α- and 5β-androstane-3α,17β-diol) and decreased excretions of conjugates of epitestosterone (ET) and its precursor androgen 5-androstene-3β,17α-diol. A dose-dependent decrease of urinary LH in response to T was also observed. The ratio (T/ET)G, which is the sole official criterium for assessment of T self-administration by athletes, increased above the threshold value of 6 in most of the subjects, but not all, after the two highest dosage T regimens, and returned to normal during the recovery period. False positive or negative testing emphasizes the need for improvement of testing procedures. In this regard, valuable complementary information may be gained from ratios such as TG/ET(Total), TG/LH, (T/5-androstene-3β,17α-diol)G, (5α/5β)androstane-3α,17α-diol and (5α/5β)androstane-3α,17β-diol.

INTRODUCTION

Most of the studies of chronic testosterone (T) administration to normal and hypogonadal men have been concerned with its effects on serum T, estradiol and gonadotropins [1–4], and on sperm production [4]. In order to gain information on the metabolic conversions of androgens in man through the analysis of urinary metabolites, the first experiments with high dose infusions of androgens were performed in the early 60s [5, 6]. Further interest for urinary androgen metabolites, excreted upon pharmacological loading with various androgen preparations, came from sports authorities faced with the problem of testing for androgen doping [7].

Xenobiotic androgens are rather easily detected by qualitative analyses using gas chromatography-mass spectrometry (GC-MS) [8, 9], but detection of exogenous T, administered as orally active or injectable preparations, is much more critical and necessitates a quantitative procedure. Moreover, as untimed urine samples have to be processed for doping control, characteristic relative excretions were sought such as the ratios (T/ET)G† (ET; epitestosterone, 17α-hydroxy-4-androsten-3-one), and also TG/LH† [10]. The former ratio and its critical value of 6 have been adopted by the International Olympic Committee for its accredited laboratories as the sole test for illicit T self-administration, according to work done by Donike *et al.* [11].

The effect of administration of T esters on the (T/ET)G and TG/LH ratios have been studied

*To whom correspondence should be addressed.

†Reports concerned with doping analysis often use T/LH and T/ET as abbreviations, where T and ET include glucuro- and nonconjugated androgen. Throughout this text, and to avoid confusion with sulfoconjugate excretion (S), TG/LH and (T/ET)G will be used, where (G) includes glucuro- and nonconjugated androgen. All the other androgen excretions include glucuronides together with minor amounts of nonconjugated parent steroid.

USADA1180

03/27/2002 16:01 FAX 7196331518 HOLME ROBERTS & OWEN 016

in some individual cases and for a single administration or for short-term administration periods [12, 13]. Our study now presents additional information on the modification of urinary excretions of various androgen metabolites during chronic high dosage T administration.

EXPERIMENTAL

Subjects and Administration Protocol

All men (mean age 29 ± 1 yr) had normal medical history, physical examination, hematological and blood chemistry studies, basal serum T, LH and FSH levels, and seminal fluid analyses which were performed as described previously [4].

Control period

This was the first 4–6 months of the study during which no hormones were administered.

Drug administration period

This period immediately followed the control period and had a length of 6 months during which one of the following regimens was administered: sesame oil vehicle (placebo) 1 ml ($n = 11$); and T enanthate (TE, Delatestryl, Squibb & Sons, Princeton, NJ, U.S.A.) 25 mg TE (= 18 mg T, $n = 7$), 100 mg TE (= 72 mg T, $n = 9$) or 300 mg TE (= 216 mg T, $n = 7$), all i.m. weekly.

Recovery period

This period corresponds to an average of 75 days (± 10 days) following the discontinuation of the TE administration period, during which no hormones were taken.

Urinalyses

Timed (3 h) urine samples were collected in the morning (between 8 and 12 a.m.) at the end of each study period (6 ± 1 days after the last TE administration). Samples of 20 ml were immediately frozen and stored at −20°C until analysis.

GC-MS with Stable Isotope Dilution

Instrumentation

GC was performed on a fused silica capillary column (25 m × 0.32 mm i.d.) coated with OV-1701 stationary phase (film thickness 0.20 μm), installed in the oven of a gas chromatograph, equipped with a glass solid injection system and heated at an isothermal temperature (between 220 and 240°C). The column was directly coupled to the source (electron impact mode) of a quadrupole mass spectrometer (Model 1010T, Nermag, Argenteuil, France) which was operated under normal ionization and mass filter settings. Data processing was performed with a PDP 11 computer (Digital Equipment France, Rungis, France) and Sidar software (Nermag).

Analytical procedures

Glucuronide fraction. In this fraction glucuronides were estimated without preliminary separation of nonconjugated androgens. The latter represent <3% of the corresponding glucuronides and remain within the experimental error range of the quantitative glucuronide estimations by selected ion monitoring (SIM). Concentrations are expressed in equivalents of nonconjugated steroid. When explicitly mentioned, nonconjugated T and ET were analyzed separately on a 1 ml urine sample as outlined for glucuronides, without the hydrolysis step.

T and ET. Enzyme-hydrolysis was performed with 10 IU (or 2000 Fishman units) of β-glucuronidase from *Escherichia coli* (Diagnostics Pasteur, Marnes-la-Coquette, France) in 0.2 ml urine (or less for concentrations higher than 10 ng/ml) diluted with 0.5 ml phosphate buffer 0.2 M, pH 6.8, and incubated for at least 4 h at 37°C, or at room temperature overnight. Stable isotope labeled analogs, [1α,2α-$2H_2$]ET synthesized as reported previously [14] and [3,4-^{13}C]T (Oris-CEA, Gif-sur-Yvette, France), were added as known amounts close to the endogenous amounts present in the sample. A mixture of *n*-hexane–diethyl ether 4:1 (v/v) was used for extraction and the residue was purified by liquid chromatography on (200 × 5 mm). Sephadex LH-20 columns, packed in the eluent which was a mixture of dichloromethane–methanol 95:5 (v/v): the first 4 ml were discarded and the next 1 ml contained T and ET. After evaporation to dryness, they were converted to the bis(heptafluorobutyrates) as described previously [15]. Reagents were evaporated under nitrogen, the residue was taken up in 30 μl of iso-octane and 3 or 6 μl were deposited on the needle of the solid injection system. SIM was performed at nominal masses *m/z* 680 and 682, and quantitative results were calculated according to equations outlined previously [16].

USADA1181

Table 1. Urinary excretion rates of LH, ET and T, at the end of different periods of the administration protocol

	ETG	ETS	TG	TS	ETNC	TNC	LH
Control ($n = 34$)							
Mean	2.37	1.40	2.47	0.65	0.021	0.058	1.27
SEM	0.33	0.17	0.41	0.13	0.003	0.021	0.15
Range	0.23–8.49	0.19–4.06	0.19–11.03	0.09–4.06	0.011–0.092	0.012–0.090	0.14–4.26
18 mg T ($n = 7$)							
Mean	1.53	0.92	1.46	0.56	0.012	0.031	0.89
SEM	0.52	0.14	0.39	0.20	0.002	0.012	0.16
Range	0.42–4.55	0.61–1.50	0.38–3.25	0.17–1.65	0.001–0.075	0.010–0.068	0.38–1.46
72 mg T ($n = 9$)							
Mean	0.36*	0.27*	3.37	0.27	0.005	0.033	0.44*
SEM	0.11	0.06	0.69	0.06	0.001	0.019	0.07
Range	0.17–1.22	0.04–0.59	0.12–6.97	0.06–0.51	0.001–0.080	0.012–0.101	0.15–0.85
216 mg T ($n = 7$)							
Mean	0.31*	0.20*	16.24	2.03	0.008	0.43*	0.36*
SEM	0.06	0.06	8.92	1.46	0.002	0.08	0.06
Range	0.06–5.77	0.02–0.45	0.50–68.07	0.04–10.77	0.001–0.066	0.06–0.69	0.17–0.56
Recovery ($n = 11$)					ND	ND	
Mean	1.22	1.21	1.46	0.35			0.95
SEM	0.21	0.26	0.36	0.06			0.20
Range	0.24–2.29	0.12–2.80	0.13–4.76	0.08–0.67			0.30–2.72

Doses of TE are given in equivalents of T. The urinary excretion rate of LH is expressed in IU/h, the others in μg free steroid/h. Control values were obtained from subjects of all treatment groups during the control period. Recovery values were obtained from subjects to whom one of the two highest T doses had been administered before the recovery period (the two groups were pooled since differences between groups were nonsignificant). *$P < 0.05$ vs control values. G = (glucuronide + nonconjugated), S = sulfate, NC = nonconjugated, ND = not determined.

Androsterone (A), etiocholanolone (E), androstanediols and androstenediols. These glucuronides, present in 0.2 ml urine, were hydrolyzed as described for T but with a longer incubation time (20 h at 37°C), and the following isotopic internal standards were added: [16,16-$^2H_2$]A, [16,16,17-$^2H_3$]5α-androstane-3α,17β-diol and [16,16,17-$^2H_3$]5-androstene-3β,17β-diol [14]. Extraction was performed with a mixture of *n*-hexane–diethyl ether 3:2 (v/v) and the residue was purified with the same chromatographic system as the one used for T: the first 4 ml were discarded, (A + E) were eluted in the next 1 ml and (androstanediols + androstenediols) thereafter with 2.5 ml eluent. (A + E) were derivatized into the heptafluorobutyrates and quantified by SIM at nominal masses *m/z* 486 and 488. 5α-Androstane-3α,17α-diol (5α-AD3α17α), 5β-androstane-3α,17α-diol (5β-AD3α17α), 5α-androstane-3α, 17β-diol (5α-AD3α17β), 5β-androstane-3α,17β-diol (5β-AD3α17β), 5-androstene-3β,17α-diol (5AD3β17α) and 5-androstene-3β,17β-diol (5AD3β17β) were converted to the bis(*t*-butyldimethylsilyl ether) as described previously [17] and quantification was performed by SIM at nominal masses *m/z* 463 and 466 (androstanediols), and *m/z* 461 and 464 (androstenediols).

Sulfate fraction. In this fraction, the sulfates of T(TS) and ET(ETS) were analyzed with preliminary separation of the nonconjugated compounds. Concentrations are expressed in equivalents of nonconjugated steroid. The internal standard [3,4-^{13}C]T 17-sulfate was synthesized by esterification of [3,4-^{13}C]T with chlorosulfonic acid [18] and purified successively by Amberlite XAD-2 chromatography and anion exchange chromatography on DEAE-cellulose. To 1 ml urine were added 20 ng labeled TS, 0.1 ml acetate buffer 1.5 M, pH 3.0, and 3 ml methanol. This mixture was then fractionated on small columns (20 × 5 mm) of DEAE-Sephadex (chloride form) packed in methanol. Glucuro- and nonconjugated steroids were washed from the column with 4 ml methanol and sulfates were eluted with 0.3 M LiCl in methanol: 0.5 ml was first discarded and the next 1.5 ml contained sulfates. Solvolysis was carried out by dissolving the dry residue in 1 ml 0.5 M HCl in methanol and heating at 60°C for 4 h. Neutralization with 0.5 ml of a saturated aqueous $NaHCO_3$ solution, evaporation of most methanol and extraction with a mixture of *n*-hexane–diethyl ether 4:1 (v/v) gave upon evaporation a dry residue which was taken-up in 0.2 ml chromatography eluent and purified by Sephadex LH-20 chromatography as described above. Quantification was performed by SIM of the bis(heptafluorobutyrate) derivatives at nominal masses *m/z* 680 and 682.

The internal standard [1α,2α-$^2H_2$]ET 17-sulfate was not suitable for use under solvolysis conditions, since prohibitive exchange of the 2α-deuterium for protium occurred. Therefore [3,4-^{13}C]T 17-sulfate was used for both TS and ETS determinations but, while standard additions of TS were quantitatively recovered,

Table 2. Urinary excretion rates of A, E, androstanediols and 5-androstenediols (glucuro- and nonconjugated) at the end of different periods of the administration protocol, expressed in μg free steroid/h

	A	E	5α-AD3α17α	5β-AD3α17α	5α-AD3α17β	5β-AD3α17β	5AD3β17α	5AD3β17β
Control ($n = 34$)								
Mean	174	193	3.01	0.31	4.74	15.56	6.09	0.42
SEM	24	23	0.51	0.04	0.46	2.16	0.85	0.05
Range	31–674	53–563	0.34–10.93	0.06–0.98	0.80–10.90	1.62–59.96	1.25–26.04	0.04–1.48
18 mg T ($n = 7$)								
Mean	131	188	2.94	0.26	2.98	11.16	5.14	0.29
SEM	22	56	0.92	0.08	0.52	2.03	1.02	0.04
Range	69–202	76–500	0.79–6.58	0.08–0.59	1.80–4.93	3.22–18.38	1.72–10.15	0.12–0.50
72 mg T ($n = 9$)								
Mean	159	205	2.47	0.24	5.40	19.78	1.75*	0.28
SEM	22	32	0.64	0.05	0.76	3.53	0.10	0.04
Range	73–277	42–376	0.40–5.69	0.02–0.46	1.05–8.43	3.20–33.90	1.29–2.16	0.11–0.49
216 mg T ($n = 7$)								
Mean	349*	454*	1.71	0.24	12.36*	49.94*	1.72*	0.29
SEM	73	94	0.56	0.09	3.09	17.19	0.40	0.09
Range	80–617	77–750	0.15–4.64	0.05–0.72	2.07–24.90	2.57–142.8	0.11–2.91	0.01–0.66
Recovery ($n = 11$)								
Mean	113	114	6.11*	0.49*	2.44*	28.33*	3.91	0.20
SEM	13	12	1.78	0.15	0.36	7.08	0.40	0.03
Range	55.3–191	51.5–161	0.89–21.0	0.11–1.80	1.12–4.91	5.15–75.22	1.88–5.80	0.09–0.58

Doses of TE are given in equivalents of T.
Control values were obtained from subjects of all treatment groups during the control period. Recovery values were obtained from subjects to whom one of the two highest T doses had been administered before the recovery period. *$P < 0.05$ vs control values.

those of ETS had an average yield of 85 ± 1% ($n = 6$). This is due to Wagner–Meerwein rearrangement catalyzed by the acidic solvolysis medium, and therefore quantitative results had to be corrected. Enzyme hydrolysis of sulfoconjugates of ET and T is not feasible by *Helix pomatia* preparations since these always contain 3β-hydroxysteroid dehydrogenase/4,5-isomerase which converts 5AD3β17α and its 17β-epimer into ET and T, respectively [19].

Accuracy and precision. The accurancy of GC-MS determinations with stable isotope dilution is principally based on permanent correction of procedural losses, on the specificities attained by the high resolution GC column and the selected ion detection, and on the purity of the steroids used as primary standards. For glucuronide determinations, standard additions of TG were made and compared with those of equivalent amounts of nonconjugated T: no significant differences were observed, thus indicating completeness of the enzyme hydrolysis step. As expected, precision was better in those cases where the corresponding isotopically labeled analogs were available. The coefficient of variation (CV) for inter-assay replicates ($n = 10$) was between 3 and 4%. In cases where an isomeric internal standard had to be used, variability increased significantly (CV was between 6 and 9%).

Radioimmunoassay

Urinary LH was measured in the Endocrine Core Laboratory of the Milton S. Hershey Medical Center (Hershey, PA, U.S.A.), according to Kulin and Santner [20] using as a standard the 2nd International Reference Preparation of hMG. Storage time of urine samples at −20°C before LH analyses did not exceed 6 months.

Statistical Analysis

Mean urinary excretion rates and their ratios were compared by Mann–Whitney U-test and correlations were established with Spearman's rank correlation coefficient, both at the $P < 0.05$ significance level.

RESULTS

As only 3 h-urine collections were available, excretions are given in μg/h. To some extent this allows comparison with μg/h-excretion values calculated from 24 h-collections, if it is taken into account that diurnal variation of androgen production leads to somewhat higher excretion rates in mid-day urine collections. Expression of urinary excretions in μg/g creatinine may be inaccurate in the case of long-term administration of anabolic steroids, which promote nitrogen retention and consequently lowers nitrogen output in the urine [21]. It was confirmed for the two highest dosage T administrations, that creatinine excretion was not significantly higher after drug administration.

Table 1 gives an overview of the different excretion rates of T and ET (free and conjugated), and also of LH, during the successive periods of the administration protocol (placebo

Table 3. Characteristic ratios of urinary excretions at the end of different periods of the administration protocol

	(T/ET)G	TG/ET (Total)	TG/LH	A/E	(5α/5β) AD3α17β	(5α/5β) AD3α17α	(ET/ 5AD3β17α)G	(T/ 5AD3β17α)G
Control ($n = 34$)								
Mean	1.38	0.74	8.31	0.89	0.39	10.20	0.43	0.51
SEM	0.22	0.09	1.51	0.08	0.04	0.88	0.05	0.10
Range	0.08–6.33	0.06–1.88	0.51–44.0	0.15–2.00	0.11–0.96	3.42–21.73	0.10–1.19	0.04–3.50
18 mg T ($n = 7$)								
Mean	1.56	0.77	7.03	0.89	0.34	11.06	0.37	0.41
SEM	0.48	0.21	2.14	0.16	0.08	1.35	0.10	0.15
Range	0.13–3.41	0.10–1.37	1.07–15.7	0.36–1.44	0.12–0.66	6.77–17.38	0.13–0.83	0.09–0.93
72 mg T ($n = 9$)								
Mean	13.35*	9.04*	39.43*	0.90	0.32	12.15	0.20*	2.02*
SEM	4.89	4.13	16.35	0.12	0.05	1.98	0.07	0.55
Range	0.66–41.88	0.21–40.80	1.00–165	0.34–1.52	0.12–0.65	3.59–20.0	0.10–0.30	0.07–5.40
216 mg T ($n = 7$)								
Mean	45.74*	30.54*	84.14*	0.83	0.40	9.08	0.24*	8.51*
SEM	21.32	14.59	22.66	0.09	0.10	2.73	0.06	3.06
Range	8.06–170.2	4.22–114.0	10.5–180	0.40–1.14	0.17–0.70	2.50–19.19	0.13–0.58	2.22–25.20
Recovery ($n = 11$)								
Mean	1.72	0.72	6.61	1.08	0.14*	20.71*	0.32	0.38
SEM	0.44	0.18	1.36	0.16	0.03	5.42	0.05	0.08
Range	0.10–3.89	0.06–1.68	0.75–14.73	0.47–2.46	0.03–0.33	3.35–50.9	0.09–0.58	0.04–0.94

Doses of TE are given in equivalents of T. TG/LH is expressed in nmol/IU.
*$P < 0.05$ vs control values. G = (glucuronide + nonconjugated), Total = (glucuronide + sulfate + nonconjugated).

values are not shown because they are, as the values obtained after administratrion of 18 mg T/week, not significantly different from control values). The low levels of nonconjugated androgens in the control period are comparable to those reported by Andino *et al.* [22], suggesting that there was adequate conservation of urine samples without spontaneous hydrolysis of conjugates. Weekly administration of 18 mg T, which corresponds roughly to a supplementation of 50% of the average endogenous T production in normal men, has no effect on the urinary androgen output. The two highest dosage T administrations induce a significant and proportionally similar decrease of both ET conjugates; urinary LH excretion is reduced to a lesser extent. Conversely, the increase of both urinary T conjugates is not significant, mainly because of the down-regulation of endogenous T production by the testes, but also by the wide variability of individual excretion values. Sulfoconjugates of T and ET are always less abundant than the corresponding glucuroconjugates, exogenous T being preferentially converted into the glucuronide.

Excretion data of other androgen metabolites are presented in Table 2: the highest dosage T administration increases T metabolites, such as A, E, 5α- and 5β-AD3α17β glucuronides. Variations of 5AD3β17β excretions are not significant, but the 17α-epimer is down-regulated similarly to ETG and ETS.

As can be anticipated from the individual excretion rates, the two characteristic excretion ratios (T/ET)G and TG/LH increase significantly after the two highest dosage T administrations (Table 3). The TG/ET(Total) ratios, which integrate ETG and ETS excretions, and the (T/5AD3β17α)G ratios have patterns similar to (T/ET)G. Some other ratios remain roughly unaffected by T administration, while (ET/5AD3β17α)G declines. The 5α/5β ratios of T metabolites, such as A/E and (5α/5β)AD3α17β have mostly values below 1, the reverse is true for the corresponding ratios of ET metabolites.

Most of the analyzed parameters reach control values after the recovery period, for the subjects to whom one of the two highest T doses (72 or 216 mg T/week) had been administered. Significant differences between control and recovery values are however observed for excretion rates of androstanediols and their corresponding ratios (Tables 2 and 3).

Among the correlations mentioned in Table 4, the most relevant ones are: between the excretion rates of each of the ET conjugates and 5AD3β17α, confirming the close relationship

Table 4. Significant correlations between various urinary excretions and excretion ratios during the control period and after administration of 72 and 216 mg T/week

	Correlation coefficients		
	Control ($n = 34$)	72 mg T ($n = 9$)	216 mg T ($n = 7$)
TG vs TS	0.79		0.98
ETS vs TS	0.72		
5α-AD3α17β vs 5β-AD3α17β	0.67		0.89
TG vs 5α-AD3α17β	0.61		
TG vs 5β-AD3α17β	0.58	0.71	0.86
ETG vs 5AD3β17α	0.58		
ETG vs ETS	0.55		
A/E vs (5α/5β)17β	0.53	0.93	0.73
ETS vs 5AD3β17α	0.51	0.90	0.84

For abbreviations see text. Missing data are nonsignificant.

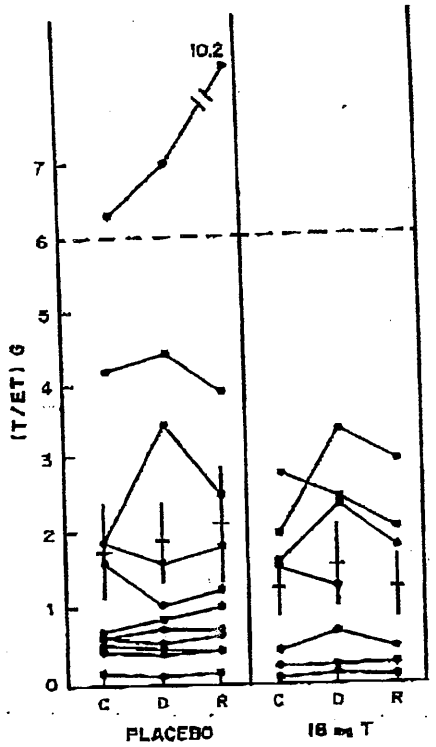


Fig. 1. Variations of the (T/ET)G ratio in different subjects, at the end of the periods of control (C), drug (D) administration (18 mg T/week) and recovery (R). Means ± SEM are indicated. (T/ET)G = 6 is the critical value above which T administration is considered as proven by sports authorities.

between ET and its precursor 5AD3β17α; between the conjugates of T and those of ET, indicating rather stable conversion rates of the parent androgens to their conjugates, with the exception of the correlation between TG and ETG which is poor ($r = 0.32$) and may thus contribute to some of the abnormal (T/ET)G ratios shown on Fig. 1. The other correlations are more or less expected on the basis of present knowledge of T metabolism in man. In particular the most specific metabolites of exogenous T (TG, 5α- and 5β-AD3α17β), are highly correlated after the highest dosage T administration.

Administration of placebo or 18 mg T/week has no effect on the average (T/ET)G ratios which remains similar to control values (Fig. 1). One subject, who received placebo, may be considered as a "false positive" with regard to the critical value of 6 for (T/ET)G. Figure 2 illustrates the increase of (T/ET)G ratios after high dosage T administrations. Although significantly increased during treatment, two subjects may be considered as "false negatives" because of (T/ET)G < 6, despite high dosage T. Return to control (T/ET)G values are generally observed during the recovery period.

DISCUSSION

With chronic high dosage T administration to normal men, Matsumoto [4] has shown that serum T increases in a dose-dependent fashion up to supraphysiological levels. A very closely related evolution of the corresponding urinary excretions of T metabolites is observed in this study. Conversely, high dosage T administration results in a dose-dependent suppression of both serum and urinary LH. The inhibition of the hypothalamo–pituitary–testicular axis with suppressed plasma levels of T and LH has been clearly demonstrated after administration of a single supraphysiological dose of deuterium-labeled T, which allowed the differentiation of the decrease of endogenous T from the increase of exogenous T [23].

With the lowest dosage T administration, neither serum T [4] nor urinary T metabolite

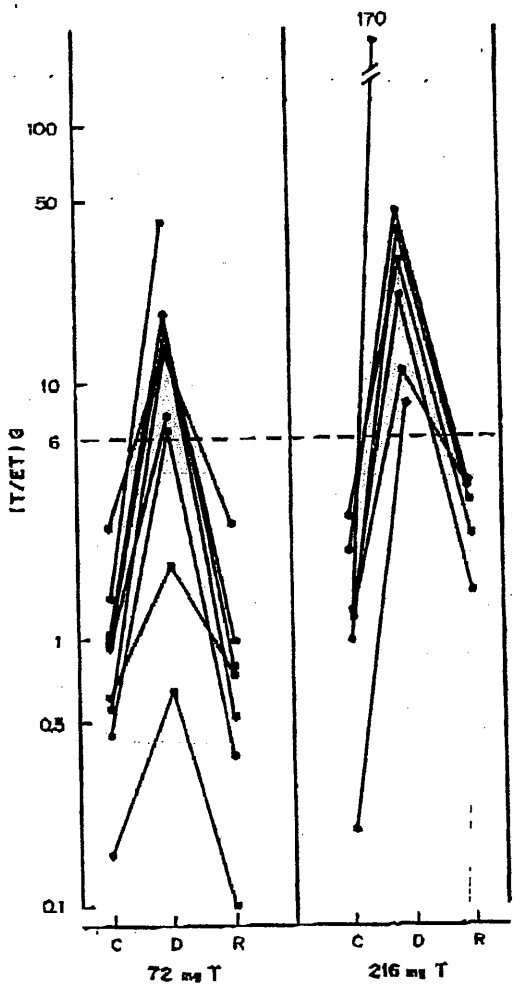


Fig. 2. Variations of the (T/ET)G ratio in different subjects, at the end of the periods of control (C), drug (D) administration and the recovery (R). Administered doses of T correspond to respectively 72 and 216 mg/week.

levels are different from control values, suggesting that the exogenous T is just able to compensate for decreased production of endogenous T. It constitutes a lower limit to the detection of T misuse in sports, although the myotropic effect of such low T supplementation is doubtful and will probably not introduce unfair competition. The (T/ET)G ratio attains the critical value of 6 under the present experimental conditions after an administration of 72 mg T/week. Based on linear interpolation between the 18 and 72 mg T/week doses, it is calculated that the threshold to achieve (T/ET)G $\geq$ 6 would be an average T dose of 47 mg T/week. This means that the average T production of normal men (about 45 mg/week) has to be doubled before testing becomes positive. Even at a dosage of 72 mg T/week two subjects failed to exceed the threshold value (Fig. 2) and this confirms earlier observations made by Friedl *et al.* [3].

The most important finding of this study is the dose-dependent down-regulation of ET production by T, which is clearly shown by the decreased excretion of both ET conjugates on high dosage T. A similar observation was made for ETG alone, after long-term administration of methandienone, a synthetic anabolic steroid [24]. On the basis of data in this study, and of other results indicating a concentration gradient of ET between peripheral and spermatic vein blood [25], it is likely that the testis is a site of ET production. Indirect evidence of both gonadal and adrenal origins of ET was offered by the increased ETG excretion after hCG and ACTH stimulation [26, 27]. Moreover, a circannual rhythm of ETG excretion has been advocated as a further proof of an adrenocortical origin for ET [28].

Although the excretion of TG represents only 1% of the metabolism of endogenous T, the highest percentage increase is observed for this specific T metabolite, and also for nonconjugated T, after chronic T administration. The increase of the (T/ET)G ratio after high dosage T administration results from an elevation of TG excretion and a fall of ETG output. The latter decrease supports the finding of an absence of peripheral conversion of T into ET [26]. However, there is a considerable difference between the average production rates of ET and T in man, with an ET production being 30 times less than T production [26]. Nevertheless, the urinary excretions of the corresponding glucuronides are similar. This may be attributed to poor metabolism of ET leading principally to renal clearance as ETG [29], and to more extensive catabolism of T.

Urinary excretions of ETS and TS are lower than the corresponding glucuronides despite the multiple origins of these sulfates: hepatic sulfoconjugation of circulating free steroids, gonadal secretion of TS [30] and ETS [25], some minute peripheral conversion of plasma T to TS [31] and a possible, but not yet proven, adrenal secretion of ETS and TS. This is in keeping with data reported by Deslypere *et al.* [32] on the higher conversion rate of a tracer dose of T to glucuronides rather than to sulfoconjugated metabolites, and also with a lower renal clearance rate and a higher biliary excretion of sulfates. The average ratio of basal TS over ETS excretions is lower than 1, in contrast to what is found for the corresponding testicular secretions [25] and this might be related to peripheral sulfatase action and direct metabolism of TS without deconjugation [33], while ETS is devoid of further metabolic conversions [29]. On the basis of the observed average urinary excretions of ETS and TS in the control period and of the percentage excretions of plasma ET and T as their sulfates, respectively 2 [34] and 0.036% [35], it can be concluded that about 15% of both urinary excretions are due to conjugation of circulating ET and T, the other 85% are then mainly attributed to a direct glandular origin.

As in one individual we studied, ratios (T/ET)G $\geq$ 6 found in some normal subjects free of any exogenous androgen [36–38] have various probabilities of occurrence depending upon the type of population studied [12, 39]. Our findings suggest that ETS excretion should also be taken into account, as is done in the TG/ET(Total) ratio (Table 3). Indeed, ETG and ETS are among the principal metabolites of ET and some changes, either in the relative hepatic conjugation of ET to each of these conjugates, or in the relative testicular secretion of ET and ETS [25], may contribute to abnormal (T/ET)G values. This is illustrated by an individual in the placebo group with an abnormally high (T/ET)G ratio (Fig. 1). When ETS excretion is taken into account in this subject, then the corresponding TG/ET(Total) ratios during control, drug administration and recovery periods remain normal (1.88, 1.36 and 1.82, respectively), if a threshold of 2.85 is adopted, which corresponds to the mean control value (0.74)

plus a confidence interval of 4 SD (2.11), similarly to what has been established officially for (T/ET)G. Moreover, the corresponding TG/LH value for this individual during treatment is not significantly different from the control value.

The basal excretions of nonconjugated T are low, but a significant increase occurs after the highest dosage T administration. This agrees with data reported after T infusion and suggests that urinary nonconjugated androgen levels are related to plasma concentrations [22].

Control values of urinary LH excretions are in agreement with those found in adult males by Kulin and Santner [20] and with those obtained with 24-h urine collections [40]. Based on the negative feedback of exogenous T on LH production, the ratio of total urinary T (obtained after hydrolysis of conjugates with a mixed β-glucuronidase/sulfatase preparation from *Helix pomatia*) over urinary LH to detect exogenous T use was first suggested by Brooks *et al.* [10] and further investigated by their colleagues at King's College [12, 41]. Indeed our mean values of TG/LH obtained after the two highest dosages of T administration are significantly more elevated than control values, but lower than those reported by Kicman *et al.* [12]. This argues for the necessity of standardizing rigorously the analytical conditions for the measurement of this ratio before establishment of any official dope testing threshold.

As expected, androsterone and etiocholanolone glucuronides are poor markers of T administration since a requisite, before excretion, is the peripheral conversion of exogenous T to androstenedione. Moreover, their ratio is remarkably constant and remains close to 1. In the control period, excretions of 5α- and 5β-AD3α17β and the corresponding (5α/5β)AD3α17β ratio are similar to reported values obtained from 24-h urine collections [42, 43]. Comparing the excretions of these androstanediols in the control period and after the highest dosage T administration, it is found that exogenous T is converted similarly to the 5β-isomer (by hepatic metabolism) as to the 5α-isomer (by mixed peripheral and hepatic metabolism) [42]. This is also corroborated by the significant correlations between the excretions of these two isomers and also between the ratios A/E and (5α/5β)AD3α17β (Table 4).

Endogenous ET is preferentially metabolized to ETG and 5α-AD3α17α, and this remains so even after T administration. Moreover, the dose-dependent decrease of excretion of ET conjugates by T is not found with the corresponding 5α- and 5β-AD3α17α glucuronides. This may be due to the fact that 5α- and 5β-AD3α17α are not exclusive metabolites of ET and that the expected decrease of these androstanediols, as a consequence of the suppression of ET production, might be compensated by some minor conversions due to the action of 17α-hydroxysteroid dehydrogenase on 17-ketosteroids. In this respect, it should be noticed that control values of 5α-AD3α17α excretions are close to those of ETG; yet they represent more than 5% conversion of circulating ET, estimated by Wilson and Lipsett [26]. Metabolism of ET by 5α-reduction is preferential to 5β-reduction, in contrast to what is observed for T.

Androstanediol excretions and their corresponding ratios, (5α/5β)AD3α17α and (5α/5β)AD3α17β, seem to be long-acting markers of high dosage T administrations. Eleven weeks after discontinuation of such T regimens there still remain some significant changes in peripheral and hepatic T and ET metabolism, leading to altered urinary androstanediol excretions.

In previous studies, the urinary excretions of 5AD3β17β and its 17α-epimer have been measured by simultaneous hydrolysis of glucuro- and sulfoconjugates with *Helix pomatia* enzyme preparations [44] and more specifically by solvolysis of sulfates [45]. We have been interested solely in glucuronides and have found that the suppression of 5AD3β17α is proportionally smaller than for ETG after the two highest dosage T administrations. Therefore, Table 3 demonstrates a decrease of the corresponding (ET/5AD3β17α)G ratios. The decline of 5AD3β17β excretions after T administration is not significant, and some conversion of exogenous T probably compensates for testicular suppression. These findings are in agreement with previous work done by Berthou [46] who demonstrated increased excretion of both conjugates of 5AD3β17α after dexamethasone inhibition of adrenal steroidogenesis and simultaneous hCG stimulation of the testis. A definitive proof of 5AD3β17α secretion by the testis came from the measurement of a significant concentration gradient between blood of peripheral and spermatic veins [25]. Convincing arguments have been presented by Weusten *et al.* [47] for a biosynthetic pathway in the testis, leading to 5AD3β17α, which is a

substrate for 3β-hydroxysteroid dehydrogenase/4,5-isomerase. This is supported here by the significant correlation between excretions of 5AD3β17α and the two ET conjugates. The estimation of the urinary excretion of 5AD3β17α may be useful in cases where the joint misuse of T and ET is suspected. The ratios (ET/5AD3β17α)G and (T/5AD3β17α)G will be increased, while (T/ET)G will remain normal [12].

Pharmacokinetic studies with a single i.m. dose of TE (100–300 mg range) administered to normal human males have demonstrated that maximal plasma levels of T are reached 10 h after injection, and that normal range plasma levels are recovered 12 days later [48]. A similar evolution has been demonstrated for the urinary (T/ET)G ratio [12]. Although the subjects of our study received multiple doses of TE (an average of 24 doses), a normal urinary profile of androgen metabolites was found at 75 (±10) days after the last TE injection. This supports the reversibility of the negative feedback regulation of LH production by exogenous T and some other anabolic androgens [49]. The minimum delay required for recovery of the basal situation after long-term administration of TE is however unknown, but it is shorter than the recovery period needed to attain control values of plasma T levels after drug withdrawal from stacked regimens with several androgenic–anabolic steroids taken by power athletes [50].

The reason why two individuals failed to exceed the critical value of 6 for (T/ET)G after administration of 72 mg T/week (Fig. 2) is unknown. It can be noticed that in such cases basal (T/ET)G is low, and that the increase of that ratio upon T administration is mainly due to depressed ETG excretion, without significant increase of TG output. This indicates that the negative feedback of exogenous T on the hypothalamo–pituitary–testicular axis is effective. The preferential urinary excretion of some glucosiduronic metabolite(s) of T other than TG is unlikely, since the excretions of all the T metabolites analyzed in this study were low. The possibility of another preferential way (bile) or/and another preferential conjugate (sulfate) for excretion will have to be investigated.

In conclusion, this study has demonstrated that chronic high dosage T administration leads to some profound changes in the urinary androgen profile, some of which should be exploited for a better assessment of T self-administration in sports. It is advisable that in cases where (T/ET)G > 6, the excretion levels of ETS, 5AD3β17α, 5α- and 5β-AD3α17α, and 5α- and 5β-AD3α17β should be evaluated. When reliable threshold values for the ratios TG/ET(Total), (T/5AD3β17α)G, (ET/5AD3β17α)G, (5α/5β)AD3α17α and (5α/5β)AD3α17β will have been established on a large number of normal (sports) men free of any anabolic steroid misuse, then they may prove to be useful adjuncts in the evaluation of surreptitious T administration in athletics.

Acknowledgements—This work was supported by US Environmental Protection Agency Grant DW-36931233, NIH Grant HD-12629 and VA Medical Research Funds in Seattle, and by Ministère de la Jeunesse et des Sports in Paris. We are indebted to Messrs. J. Dubois and P. Salvador for qualified technical aid.

REFERENCES

1. Schürmeyer T., Wickings E. J., Freischem C. W. and Nieschlag E.: Saliva and serum testosterone following oral testosterone undecanoate administration in normal and hypogonadal men. *Acta Endocr., Copenh.* **102** (1983) 456–462.
2. Sokol R. Z, Palacios A., Campfield L. A., Saul C. and Swerdloff R. S.: Comparison of the kinetics of injectable testosterone in eugonadal and hypogonadal men. *Fert. Steril.* **37** (1982) 425–430.
3. Friedl K. E., Jones R. E., Hannan C. J. and Plymate S. R.: The administration of pharmacological doses of testosterone or 19-nortestosterone to normal men is not associated with increased insulin secretion or impaired glucose tolerance. *J. Clin. Endocr. Metab.* **68** (1989) 971–975.
4. Matsumoto A. M.: Effects of chronic testosterone administration in normal men: safety and efficacy of high dosage testosterone and parallel dose dependent suppression of luteinizing hormone, follicle-stimulating hormone, and sperm production. *J. Clin. Endocr. Metab.* **70** (1990) 282–287.
5. Brooks R. V. and Giuliani G.: Epitestosterone: isolation from human urine and experiments on possible precursors. *Steroids* **4** (1964) 101–116.
6. Tamm J., Volkwein U. and Starcevic Z.: The urinary excretion of epitestosterone, testosterone and androstenedione following intravenous infusions of high doses of these steroids in human subjects. *Steroids* **8** (1966) 659–669.
7. Wilson J. D.: Androgen abuse by athletes. *Endocrine Rev.* **9** (1988) 181–199.
8. Massé R., Ayotte C. and Dugal R.: Studies on anabolic steroids. I Integrated methodological approach to the gas chromatographic-mass spectrometric analysis of anabolic steroid metabolites in urine. *J. Chromat.* **489** (1989) 23–50.
9. Hatton C. K. and Catlin D. H.: Detection of androgenic anabolic steroids in urine. *Clins. Lab. Med.* **7** (1987) 655–668.
10. Brooks R. V., Jeremiah G., Webb W. A. and Wheeler M. J.: Detection of anabolic steroid administration to athletes. *J. Steroid Biochem.* **11** (1979) 913–917.
11. Donike M., Bärwald K. R., Klostermann K., Schänzer W. and Zimmermann J.: Nachweis von exogenem Testosteron. In *Sport: Leistung und Gesundheit* (Edited by H. Heck, W. Hollmann, H. Liesen and R. Rost). Deutscher Ärtze-Verlag, Köln (1983) pp. 293–300.

12. Kicman A. T., Brooks R. V., Collyer S. C., Cowan D. A., Nanjee M. N., Southan G. J. and Wheeler M. J.: Criteria to indicate testosterone administration. *Br. J. Sports Med.* **24** (1990) 253–264.
13. de Boer D., De Jong E. G., van Rossum J. M. and Maes R. A. A.: Doping control of testosterone and human chorionic gonadotrophin. *Int. J. Sports Med.* **12** (1991) 46–51.
14. Dehennin L., Reiffsteck A. and Scholler R.: Simple methods for the synthesis of twenty different highly enriched deuterium labelled steroids suitable as internal standards for isotope dilution-mass spectrometry. *Biomed. Mass Spectr.* **7** (1980) 493–499.
15. Dehennin L., Reiffsteck A. and Scholler R.: Halogenated diesters of testosterone. Response to electron capture detection, gas chromatographic and mass spectrometric properties. *J. Chromat. Sci.* **10** (1972) 224–230.
16. Reiffsteck A., Dehennin L. and Scholler R.: Estrogens in seminal fluid of human and animal species: identification and quantitative estimation by gas chromatography-mass spectrometry associated with stable isotope dilution. *J. Steroid Biochem.* **17** (1982) 567–572.
17. Dehennin L.: Mixed silyl ether-perfluoroacyl ester derivatives for gas chromatography/mass spectrometry of œstrogens. Application to the quantitative determination of œstriol in human plasma. *Biomed. Mass Spectr.* **18** (1989) 314–320.
18. Tanaka T., Hachinoe M. and Kubodera A.: A radioimmunoassay of deoxycorticosterone sulfate without hydrolysis. *Steroids* **49** (1987) 523–530.
19. Messeri G., Cugnetto G., Moneti G. and Serio M.: *Helix pomatia* induced conversion of some 3β-hydroxysteroids. *J. Steroid Biochem.* **20** (1984) 793–796.
20. Kulin H. E. and Santner S. J.: Timed urinary gonadotropin measurements in normal infants, children, and adults, and in patients with disorders of sexual maturation. *J. Pediat.* **90** (1977) 760–765.
21. Liddle G. W. and Burke H. A. Jr: Anabolic steroids in clinical medicine. *Helv. Chim. Acta* **27** (1960) 504–513.
22. Andino N., James Y. H. T., Parker Y. and Rippon A. E.: Excretion of non-conjugated androstenedione and testosterone in human urine. *Steroids* **28** (1976) 837–846.
23. Fujioka M., Shinohara Y., Baba S., Irie M. and Inoue K.: Acute suppression of endogenous testosterone levels by exogenous testosterone in normal men. *Life Sci.* **41** (1987) 945–949.
24. Donike M., Geyer H., Kraft M. and Rauth S.: Longterm influence of anabolic steroid misuse on the steroid profile. In *Official Proceedings of 2nd International Athletic Foundation World Symposium on Doping in Sport* (Edited by P. Bellotti, G. Benzi and A. Ljungqvist). IAF Foundation, Monte Carlo (1990) pp. 107–116.
25. Dehennin L.: Secretion by the human testis of epitestosterone, with its sulfoconjugate and precursor androgen 5-androstene-3β,17α-diol. *J. Steroid Biochem. Molec. Biol.* **44** (1993) 171–177.
26. Wilson H. and Lipsett M. B.: Metabolism of epitestosterone in man. *J. Clin. Endocr. Metab.* **26** (1966) 902–914.
27. Tamm J., Apostolakis M. and Voigt K. D.: The effects of ACTH and hCG on urinary excretion of testosterone in male patients with various endocrine disorders. *Acta Endocr., Copenh.* **53** (1966) 61–72.
28. Lagoguey M., Dray F., Chauffournier J.-M. and Reinberg A.: Circadian and circannual rhythms of urinary testosterone and epitestosterone glucuronide in healthy adult men. *J. Chronobiol.* **1** (1973) 91–93.
29. Dray F.: Contribution à l'analyse des stéroïdes œstroniques et à l'étude métabolique de la testostérone et de l'épitestostérone. Thesis, University of Paris VI (1970) 110–152.
30. Laatikainen T., Laitinen E. A. and Vihko R.: Secretion of neutral steroid sulfates by human testis. *J. Clin. Endocr. Metab.* **29** (1969) 219–224.
31. Saez J. M., Bertrand J. and Migeon C. J.: Metabolic clearance rate, urinary and plasma production rates of testosterone sulfate in man. *Steroids* **17** (1971) 435–452.
32. Deslypere J.-P., Wiers P. W., Sayed A. and Vermeulen A.: Urinary excretion of androgen metabolites, comparison with excretion of radioactive metabolites after injection of [4-^{14}C]testosterone. Influence of age. *Acta Endocr., Copenh.* **96** (1981) 265–271.
33. Dessypris A. G.: Testosterone sulphate, its biosynthesis, metabolism, measurement, functions and properties. *J. Steroid Biochem.* **6** (1975) 1287–1298.
34. Dray F. and Ledru M.-J.: Métabolisme de l'épitestostérone. Absence d'interconversion périphérique de l'épitestostérone et de la testostérone et existence d'une production de sulfate d'épitestostérone chez l'homme adulte normal. *C.R. Acad. Sci. Paris, Série C* **262** (1966) 679–681.
35. Camacho A. M. and Migeon C. J.: Studies on the origin of testosterone in the urine of normal adult subjects and patients with various endocrine disorders. *J. Clin. Invest.* **43** (1964) 1083–1089.
36. Namba O., Miyachi Y., Kawahara T., Irie M. and Kuroda Y.: Urinary testosterone and epitestosterone excretion in a doping positive subject. In *Hormones and Sport, Serono Symposia Publications* (Edited by Z. Laron and A. D. Rogol). Raven Press, New York (1989) pp. 275–283.
37. Dalzell D. P. and ElAttar T. M. A.: Gas chromatographic determination of urinary excretions of testosterone, epitestosterone and androstenedione in pre-adolescent and adolescent children. *J. Clin. Endocr. Metab.* **36** (1973) 1237–1243.
38. Okamoto M., Setaischi C., Horiuchi Y., Moriya K. and Itoh S.: Urinary excretion of testosterone and epitestosterone in Japanese and Ainu. *J. Clin. Endocr. Metab.* **32** (1971) 673–674.
39. Dehennin L. and Scholler R.: Dépeistage de la prise de testostérone comme anabolisant chez les adolescents par la détermination du rapport des excrétions urinaires de testostérone et d'épitestostérone. *Path. Biol., Paris* **38** (1990) 920–922.
40. Kulin H. E., Bell P. M., Santen R. J. and Ferber A. J.: Integration of pulsatile gonadotropin secretion by timed urinary measurements: an accurate and sensitive 3-hour test. *J. Clin. Endocr. Metab.* **40** (1975) 783–789.
41. Cowan D. A., Kicman A. T., Walker C. J. and Wheeler M. J.: Effect of administration of human chorionic gonadotrophin on criteria used to assess testosterone administration in athletes. *J. Endocr.* **131** (1991) 147–154.
42. Deslypere J.-P., Sayed A., Punjabi U., Verdonck L. and Vermeulen A.: Plasma 5α-androstane-3α,17β-diol and urinary 5α-androstane-3α,17β-diol glucuronide, parameters of peripheral androgen action: a comparative study. *J. Clin. Endocr. Metab.* **54** (1982) 386–391.
43. Muller L. and Philippou G.: Quantification of 5α- and 5β-androstanediols in urine by gas chromatography-mass spectrometry. *Clin. Chem.* **33** (1987) 256–260.
44. Shackleton C. H. L., Livingstone J. R. B. and Mitchell F. L.: The conjugated 17-hydroxy epimers of Δ^5-androstene-3β,17-diol in infant and adult urine and umbilical cord plasma. *Steroids* **11** (1968) 299–311.
45. Jänne O.: Quantitative determination of neutral steroid mono- and disulfates in human urine. *Clin. Chim. Acta* **29** (1970) 529–540.

46. Berthou F.: Application de la chromatographie en phase gazeuse sur colonnes capillaires de verre à l'analyse des dihydroxystéroïdes urinaires humains. Thesis, University of Brest (1977) 184–190.
47. Weusten J. J. A. M., Legemaat G., Van der Wouw M. P. M. E., Smals A. G. H., Kloppenborg P. W. C. and Benraaad T. H.: The mechanism of the synthesis of 16-androstenes in human testicular homogenates. *J. Steroid Biochem.* 32 (1989) 689–694.
48. Behre H. M. and Nieschlag E.: Comparative pharmacokinetics of androgen preparations: applications of computer analysis and simulation. In *Testosterone. Action, Deficiency, Substitution* (Edited by E. Nieschlag and H. M. Behre). Springer-Verlag, Berlin (1990) pp. 115–135.
49. Alén M., Reinilä M. and Vihko R.: Response of serum hormones to androgen administration in power athletes. *Med. Sci. Sports Exercise* 17 (1985) 354–359.
50. Ruokonen A., Alén M., Bolton N. and Vihko R.: Response of serum testosterone and its precursor steroids, SHBG and CBG to anabolic steroid and testosterone self-administration in man. *J. Steroid Biochem.* 23 (1985) 33–38.

USADA1191

M. Donike, S. Rauth, U. Mareck-Engelke, H. Geyer and R. Nitschke

Evaluation of Longitudinal Studies, the Determination of Subject Based Reference Ranges of the Testosterone / Epitestosterone Ratio

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

The observations made in the past in measuring the steroid profile prove that for a given individual the excreted quantities of endogenous steroids are more or less constant [1]. The reason for this is the homeostasis of biosynthesis and metabolism of endogenous steroids [2, 3]. The concentrations of the eliminated endogenous steroids vary according to the urinary flow, whereas the concentration ratios of steroids are more constant [4].

The homeostasis of the endocrine steroid biosynthesis and metabolism is not disturbed by physical workload at least not to such an extend that the T/E ratio of an individual is altered [5]. The T/E ratios in longitudinal studies fluctuate around the mean with a CV which will not exceed 30%.In table 1 the T/E ratio of sport students with moderate physical activity are summarized. High work load will also not influence the T/E ratio to a greater extend. The T/E ratios of a professional cycling team participating in the Tour de France 1992 with 9 riders are presented in table 2. Training in high altitude had also no influence on the T/E ratio as we observed in following up the German rowing team before the 1988 Olympic Games in Seoul.

In case of testosterone application, the concentration of testosterone glucuronide will be increased more than any of the other testosterone metabolites leading to a significant increase of the individuals ratios of testosterone to epitestosterone [6, 7]. Depending on the route of administration, the dose, chemical structure of the ester etc. the T/E ratio will be elevated for some time before it returns to normal.

The increase of the T/E ratio is more prominent than the increase of other ratios within the steroid profile because testosterone is not metabolized to epitestosterone but contributes to androsterone and etiocholanolone [6, 7]. But nevertheless, also the ratios of testosterone to androsterone or etiocholanolone are increased and maybe used as additional parameters.

To monitor testosterone application the question to answer is, if the ratio observed in an athlete's urine is higher than "normal".

Two possible approaches to answer this question are:
1. Comparison with a **population-based** reference range [8]
2. Comparison with the **subject-based** reference range.[8, 9]

The first approach has been chosen 1982 by the IOC Medical Commission to define a decision limit for testosterone application (T/E=6) [10, 11]. This ratio discriminates testosterone users from non-users only when the epitestosterone concentration is proportional to the concentrations of the other endogenous steroids. In case of a low epitestosterone concentration a few individuals may have a T/E ratio of higher than 6. This observation led to the decision of the IOC Medical Commission to recommend a follow-up of T/E ratios between 6 and 10 by longitudinal studies, to exclude physiological reasons for an elevated T/E ratio [12].

The proposal of the IOC Medical Commission to collect data in longitudinal investigations for an athlete found to have a T/E-ratio higher than 6 and lower than 10 provides the data to calculate a subject-based reference range [9].

The necessary data may be obtained:
1. by requesting from the authorities documented T/E ratios of previous tests - retrospective data
2. by additional unannounced in and/or out of competition doping controls - prospective data
3. a study under quarantaine-like conditions.

Evaluation of the longitudinal approach

The problem can be statistically defined as the discrimination of an outlier from the series of T/E-ratios measured on the same individual. An outlier can be defined e.g. as a value lying outside the mean ±4 times the standard deviation calculated without the suspicious value.

For a consensus approach the following parameters must be defined:
1. the number of T/E ratios (in addition to the suspicious value)
2. the statistical probability
3. the time interval in which additional samples shall be collected
4. the acceptance of retrospective values
5. the definition of an outlier.

Proposal for the calculation of the subject-based reference range [9]:
1. calculate the mean out of at least 3 observations
2. calculate the limit for the outliers (l_o) following the formula below:

$$l_o = \text{mean} + t(p,n) * \text{stdev}$$

Note: All factors which may influence the analytical measurements will lead to a greater standard deviation, e.g. values determined in different laboratories or extent of physical workload. The greater standard deviation will give a higher limit for outliers and by this be in favor of the athlete.

Table 1: T/E-ratio of 5 male volunteers under resting conditions; urine samples taken in the morning [1].

cyclist	n	mean	s	c.v.%	min	max
1	30	1.82	0.21	11.4	1.41	2.20
2	30	0.13	0.01	11.0	0.11	0.16
3	30	0.91	0.10	11.0	0.79	1.22
4	30	0.90	0.15	16.2	0.69	1.46
5	30	1.04	0.17	16.0	0.76	1.53

USADA1192

USADA1193

Table 2a: T/E-ratio of 9 cyclists participating in the Tour de France 1992; urine samples collected over night.

cyclist	n	mean	s	c.v.%	min	max
1	9	1,81	0,34	18,82	1,36	2,45
2	14	0,22	0,06	27,69	0,10	0,34
3	23	2,02	0,15	7,47	1,72	2,38
5	22	1,85	0,24	13,02	1,36	2,44
6	11	0,12	0,04	38,00	0,09	0,24
7	21	1,22	0,16	12,67	0,78	1,48
8	15	0,84	0,08	9,24	0,69	0,96
9	21	1,87	0,34	18,24	1,28	2,55

Table 2b: T/E-ratio of 9 cyclists participating in the Tour de France 1992; urine samples taken after each stage.

cyclist	n	mean	s	c.v.%	min	max
1	7	2,34	0,28	12,11	1,90	2,72
2	11	0,24	0,08	31,88	0,10	0,35
3	18	2,34	0,36	15,25	1,56	2,78
5	21	2,38	0,59	24,80	1,21	3,55
6	9	0,13	0,02	19,08	0,10	0,18
7	18	1,32	0,21	15,82	0,95	1,60
8	12	0,76	0,14	18,25	0,62	1,07
9	19	1,80	0,32	17,77	1,36	2,61

Table 3: T/E-ratio of the German rowing team (males) in a training camp in high altitude preparing the Olympic Summer Games in Seoul from the 18th August till the 7th September 1988.

Urine samples have collected over a time period of about 12 hours overnight.

rower	N	mean	stdev	vc %	min	max
1	7	1,99	0,14	7,09	1,76	2,11
2	6	4,04	0,53	13,21	3,20	4,81
3	6	0,87	0,20	23,41	0,68	1,24
4	5	0,93	0,14	14,99	0,80	1,17
5	5	2,79	0,30	10,88	2,42	3,10
6	6	0,08	0,02	24,79	0,06	0,11
7	7	1,06	0,22	20,81	0,81	1,38
8	5	1,39	0,17	12,36	1,20	1,63
9	4	1,79	0,40	22,55	1,40	2,27
10	6	2,63	0,57	21,50	1,59	3,25
11	6	0,72	0,07	9,32	0,63	0,82
12	0	no data	no data	no data		
13	5	0,74	0,07	9,18	0,63	0,79
14	7	0,51	0,13	26,29	0,34	0,67
15	7	0,93	0,19	20,68	0,68	1,23
16	6	0,20	0,03	13,87	0,17	0,24
17	7	1,77	0,29	16,38	1,27	2,10
18	6	3,93	0,97	24,77	2,92	5,38
19	7	0,76	0,16	20,69	0,52	1,00
20	6	0,84	0,14	16,56	0,70	1,08
21	6	0,17	0,04	20,93	0,13	0,22
22	7	1,15	0,19	16,48	0,86	1,43
23	7	0,81	0,24	29,61	0,50	1,11
24	6	0,10	0,05	52,47	0,06	0,20

References

1. MARECK-ENGELKE, U.; GEYER, H.; DONIKE, M.: Stability of Steroid Profiles. In: *10th Cologne Workshop on Dope Analysis, 7th to 12th June 1992 - Proceedings, M. Donike, H. Geyer, A. Gotzmann, U.Mareck-Engelke und S. Rauth (Hrsg.),* Sport und Buch Strauß, Köln (1993), 87-89
2. Shackleton, C.H.L.: Profiling steroid hormones and urinary steroids. *J. Chromatogr.* 379 (1986), 91
3. VESTERGAARD, P.: Excretion of common neutral steroids in healthy subjects as estimated by multi-column chromatography. In: *Estimation after multi-column liquid chromatography of common urinary neutral steroids with an application to the assay of plasma 17-oxosteroids. Vestergaard, P.; Sayegh, J.F.; Mowat, J.H.; Hemmingsen, L. (Hrsg.), Acta Endocrin.* 88 (1978), Suppl. 217, 157-189.
4. DONIKE, M.; RAUTH, S.; SAMPLE, B.: Excretion of ephedrine and endogenous steroids under conditions of controlled water intake and of water diuresis. In: *10th Cologne Workshop on Dope Analysis, 7th to 12 th June 1992 - Proceedings. Donike, M., H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (Hrsg.),* Sport und Buch Strauß, Köln (1993), 163-176
5. GEYER, G.: Die gas-chromatographisch/massenspektrometrische Bestimmung von Steroidprofilen im Urin von Athleten Deutsche Sporthochschule Köln: Dissertation, 1990
6. ZIMMERMANN, J.: *Untersuchungen zum Nachweis von exogenen Gaben von Testosteron* Hartung-Gorre Verlag, Konstanz, Diss. Deutsche Sporthochschule Köln, 1986
7. DONIKE, M.: Steroid Profiling in Cologne. In: *10th Cologne Workshop on Dope Analysis, 7th to 12th June 1992 - Proceedings. M. Donike, H. Geyer, A. Gotzmann, U.Mareck-Engelke und S. Rauth (Hrsg.),* Sport und Buch Strauß, Köln (1993), 47-86
8. SOLBERG, H.E.; GRÄSBECK, R.: *Reference Values* Bd. 27. Advances in Clinical Chemistry. Academic Press, New York, 1989
9. HARRIS, E.K.: Some Theory of Reference Values. I. Stratified (Categorized) Normal Ranges and a Method for Following an Individual's Clinical Laboratory Values. *Clin.Chem.* 21 (1975), 10, 1457-1464
10. ADAMIETZ, B.: *Zum Problem der Applikation endogener Substanzen. Statistisches Verfahren zur Bestimmung von Normbereichen.* Deutsche Sporthochschule Köln: Examensarbeit, 1984

11. DONIKE, M.; ADAMIETZ, B.; OPFERMANN, G.; SCHÄNZER, W.; ZIMMERMANN, J.; MANDEL, F.: Die Normbereiche für Testosteron- und Epitestosteron-Urinspiegel sowie des Testosteron-/Epitestosteron-Quotienten. In: *Training und Sport zur Prävention und Rehabilitation in der technisierten Umwelt. I.-W.Franz, H.Mellerowicz, W.Noack (Hrsg.),* Springer Verlag, Berlin, Heidelberg (1985), 503-507
12. INTERNATIONAL OLYMPIC COMMITTEE, Medical COMMISSION: List of Doping Classes and Methods. 17th March 1993.

USADA1194

USADA1195

U.Mareck-Engelke, H.Geyer and M.Donike

Stability of Steroid Profiles (2) : Excretion Rates from Morning Urines

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

The excretion rates of endogenous steroids in male and female urine were investigated. In this study six male (age: $\bar{x}$=33 ±5 years) and six female subjects (age: $\bar{x}$= 32 ±4 years) participated.

Morning urine samples were collected for 30 days (men) respectively for one menstrual cycle (women).

The urines were prepared according to the screening procedure of conjugated anabolic steroids and analysed by GC/MS (1).

The following steroid glucuronides were measured: androsterone (and), etiocholanolone (etio), testosterone (t), epitestosterone (epit), 11ß-OH-androsterone (OHa), 11ß-OH-etiocholanolone (OHe), 3α5α-androstandiol (a-diol) and 3α,5ß-androstandiol (b-diol).

The results of the excretion rates and their statistical evaluation are shown in Tables 1-4.

Conclusions:

- On principle, excretion rates of morning urine samples are not constant.
- The excretion rates of androsterone, etiocholanolone, epitestosterone and testosterone of most male volunteers shows only little variation.
- In female volunteers the excretion rates of epitestosterone and testosterone shows much more variation. The concentrations of these substances are near the detection limit and frequently other endogenous substances coelute with testosterone and epitestosterone.

References

(1) Donike M., Geyer H., Gotzmann A., Kraft M., Mandel F., Nolteernsting E., Opfermann G., Sigmung G., Schänzer W. and Zimmermann J.: Dope Analysis
In: Official Proceedings of the International Athletic Foundation World Symposium on Doping in Sport. P.Bellotti, G.Benzi, A.Ljungquist (Hrsg.) IAAF Florence (1988) 53-87.

(2) Mareck-Engelke U., Geyer H. and Donike M.: Stability of steroid profiles
In: 10th Cologne Workshop On Dope Analysis, 7th To 12th June 1992 . -Proceedings-, 87-89

Table 1: Stability of steroid profiles (male urines).
Coefficient of variation (%) of excretion rates
(V1-V6 = volunteer 1-6).

	V1	V2	V3	V4	V5	V6
and	19	19	15	13	43	12
etio	15	23	15	17	42	15
epit	19	19	13	16	41	15
t	22	20	17	21	43	16
a-diol	25	40	16	27	45	16
b-diol	21	46	16	27	47	15
OHa	40	35	15	21	43	25
OHe	31	46	21	25	69	33

Table 2: Stability of steroid profiles (male urines).
Statistics of some selected excretion rates
(min = minimum value; max = maximum value;
st.de. = standard deviation; c.v. = coefficient of variation (%).

		V1	V2	V3	V4	V5	V6
and (µg/h)	min	56	41	46	114	74	86
	max	134	118	111	195	487	138
	mean	86	87	84	144	233	111
	st.dev.	17	17	12	19	101	14
	c.v. (%)	19	19	15	13	43	12
etio (µg/h)	min	38	36	49	83	47	50
	max	76	105	117	165	342	79
	mean	56	74	82	104	163	59
	st.dev.	8.5	17	12	18	68	8.7
	c.v. (%)	15	23	15	17	42	15
epit (µg/h)	min	0.9	0.8	1.1	1.6	0.5	0.5
	max	2.0	2.5	1.9	3.2	3.6	1.0
	mean	1.5	1.8	1.5	2.0	1.9	0.7
	st.dev.	2.28	0.33	0.20	0.33	0.79	0.11
	c.v. (%)	19	19	13	16	41	15
t (µg/h)	min	1.7	0.12	0.9	1.3	0.6	2.6
	max	3.8	0.35	2.1	3.3	3.4	5.4
	mean	2.6	0.23	1.4	1.8	2.0	3.9
	st.dev.	0.59	0.05	0.23	0.38	0.85	0.64
	c.v. (%)	22	20	17	21	43	16

USADA1196

Table 3: Stability of steroid profiles(female urines).
Coefficient of variation (%) of excretion rates
(V1-V6 = volunteer 1-6).

	V1	V2	V3	V4	V5	V6
and	15	21	21	14	23	28
etio	18	23	21	16	24	39
epit	30	47	35	32	28	38
t	34	25	59	44	32	40
a-diol	24	18	65	14	28	38
b-diol	43	26	47	22	28	67
OHa	29	48	36	29	28	34
OHe	44	54	25	37	44	55

Table 4: Stability of steroid profiles (female urines).
Statistics of some selected excretion rates
(min = minimum value; max = maximum value; st.de. = standard deviation;
c.v. = coefficient of variation (%).

		V1	V2	V3	V4	V5	V6
and (µg/h)	min	43	81	22	54	22	48
	max	73	161	57	96	66	151
	mean	56	112	39	71	38	74
	st.dev.	8.2	24	8.1	10.1	8.57	21
	c.v. (%)	15	21	21	14	23	28
etio (µg/h)	min	35	48	30	72	39	67
	max	73	108	72	129	113	299
	mean	49	67	52	95	66	114
	st.dev.	9.1	15	11.1	15.2	16	44
	c.v. (%)	18	23	21	16	24	39
epit (µg/h)	min	0.03	0.2	0.03	0.07	0.08	0.2
	max	0.1	1.07	0.13	0.26	0.3	0.97
	mean	0.06	0.56	0.06	0.15	0.19	0.57
	st.dev.	0.02	0.26	0.02	0.05	0.05	0.22
	c.v. (%)	30	47	35	32	28	38
t (µg/h)	min	0.13	0.23	0.06	0.02	0.19	0.36
	max	0.42	0.59	0.43	0.14	0.64	1.69
	mean	0.2	0.43	0.14	0.08	0.32	0.85
	st.dev.	0.07	0.11	0.08	0.04	0.1	0.34
	c.v. (%)	34	25	59	44	32	40

USADA1197

JOURNAL OF MASS SPECTROMETRY, VOL. 31, 169–176 (1996)

Improved Method of Detection of Testosterone Abuse by Gas Chromatography/Combustion/ Isotope Ratio Mass Spectrometry Analysis of Urinary Steroids

Rodrigue Aguilera, Michel Becchi and Hervé Casabianca
Service Central d'Analyse, CNRS, BP 22, 69390 Vernaison, France

Caroline K. Hatton, Don H. Catlin and Borislav Starcevic
UCLA Olympic Laboratory, Department of Molecular and Medical Pharmacology, University of California at Los Angeles, 2122 Granville Avenue, Los Angeles, CA 90025-6106, USA

Harrison G. Pope, Jr.
Harvard Medical School, McLean Hospital, 115 Mill Street, Belmont, MA 02178-9106, USA

The current approach to detection of doping with testosterone is based on measuring the testosterone to epitestosterone ratio (T/E) in urine by gas chromatography/mass spectrometry. The median T/E for healthy males who have not used T is about 1.0. In a single urine, a T/E lower than six leads to a negative report even though it does not exclude T administration. A value greater than six indicates possible T administration or a naturally elevated ratio. It has been shown previously that the carbon isotope ratio of urinary T changes after T administration. In this study a potential confirmation method for T abuse was optimized. Gas chromatography/combustion/carbon isotope ratio mass spectrometry (GC/C/IRMS) was used to analyze two T precursors (cholesterol and 5-androsten-3β,17β-diol) and two T metabolites (5α- and 5β-androstane-3α,17β-diol) in addition to T itself in each of 25 blind urines collected from eight healthy men before, during or after T administration. The carbon isotope ratios of T and the metabolites were lower after T administration. The relationships among the variables were studied using multivariate analysis and beginning with principal components analysis; cluster analysis revealed that the data are composed of two clusters, and classified the samples obtained after T administration in one cluster and the remainder in the other; discriminant analysis correctly identified T users. The measurement of carbon isotope ratios of urinary androgens is comparable to the T/E > 6 test and continues to show promise for resolving cases where doping with T is suspected.

KEYWORDS: testosterone; detection; isotope ratio mass spectrometry; doping control; urine

INTRODUCTION

Sport and other organizations ban the use of anabolic steroids and enforce the ban by analysis of urine by gas chromatography/mass spectrometry (GC/MS).[1–3] Testosterone (T) administration is especially difficult to detect because the current method does not distinguish synthetic exogenous T from natural endogenous T. Detection of T administration is based on the ratio of T to 17-epitestosterone (E) in urine. Since the early 1980s, the International Olympic Committee rule has stated that a urinary T/E above six constitutes an offence;[4] this remains the case today unless there is evidence that this ratio is due to a physiological or pathological condition.[5] T administration elevates the urinary T/E[6] by increasing the concentration of T and decreasing that of E.[7]

Drug tests based on the current method may miss cases with T/E not greater than six in which T (or both T and E) might have been used or lead to reporting cases in which the elevated ratio might be natural. Different approaches to distinguishing T users from nonusers include measuring the urinary ratio of T to luteinizing hormone,[8] measuring serum T/17-hydroxyprogesterone,[9] administration of the ketoconazole test,[10] measuring urinary E glucuronide/E sulfate[11] and measuring urinary T glucuronide/E glucuronide plus E sulfate.[7] Sport organizations may require an endocrine evaluation and follow-up T/E tests before adjudicating cases with a T/E greater than six.[5,12] In this paper, we explore an approach to detecting T administration based on a measurable difference in carbon isotope ratio ($^{13}C/^{12}C$) between synthetic and natural T.

We have shown previously that this carbon isotope ratio changes after administration of exogenous T.[13] This can only be explained by a difference between the natural and synthetic T. That the natural and synthetic homologs of an organic molecule may have different carbon isotope ratios has been shown for vanillin among others and exploited successfully to detect the adulteration of expensive natural vanillin with inexpen-

CCC 1076–5174/96/020169–08
© 1996 by John Wiley & Sons, Ltd.

Received 2 September 1995
Accepted 16 October 1995

USADA1198

sive synthetic vanillin.[14] Why there is a difference may be explained, at least hypothetically. All carbon in biological molecules is ultimately derived from atmospheric carbon dioxide fixed by photosynthesis. Upon uptake and inclusion of CO_2 into the first-formed organic molecule, there is isotopic fractionation and depletion in the heavier (^{13}C) isotope, probably due to enzymatic kinetic isotope effects. In the two main pathways for photosynthesis, the first-formed organic intermediate has either three or four carbons, and therefore the two pathways are referred to as 'C3' or 'C4'; they are catalyzed by different enzymes. C3 and C4 plant compounds have different ^{13}C contents. The ^{13}C content of a given organic molecule reflects the proportions of C3 and C4 plant compounds that are at the origin of its carbon atoms.[15,16]

Isotope ratios are measured by isotope ratio mass spectrometry (IRMS). The instrument measures the isotopomers of CO_2, therefore the carbon atoms in the molecule of interest must all be converted into CO_2; this is accomplished by combustion over an oxidant. A preliminary GC step is performed for a final separation of the analyte of interest from other compounds present in the biological extract. Thus we connected a gas chromatograph to a combustion interface to an isotope ratio mass spectrometer (GC/C/IRMS) (see Fig. 2).[17] GC/C/IRMS accomplishes the same chromatographic resolution as GC/MS, so it provides the same degree of compound identification based on chromatographic retention time, but it does not provide the definitive identification of a mass spectrum; instead, combustion converts all carbon present into CO_2, and the mass spectrometer acts as a non-specific detector.

EXPERIMENTAL

Urine samples

The urines were obtained from eight healthy men, aged 20–59, who were participating in a 24-week study concerned with the effects of T on behavior. The protocol was approved by the Harvard Medical School institutional review board. A double-blind, cross-over design was used with the subjects receiving either placebo or T cypionate during the first 6 weeks, nothing during the second 6 weeks, T cypionate or placebo (whichever was not received during the first 6 weeks) during the next 6 weeks and nothing during the last 6 weeks. The T cypionate preparation contained 200 mg ml^{-1} in sesame oil. During the 6 weeks of T administration the subjects received intragluteal injections of 150 mg on the first day of weeks one and two, 300 mg on the first day of weeks three and four and 600 mg on the first day of weeks five and six. Urines were collected every 3 weeks. Thus the study generated a total of 64 urine samples.

Overview of the analyses

In total, each urine underwent one or three types of analyses. First all 64 samples underwent one sample preparation followed by a GC/MS anabolic steroid screen (to estimate the T/E and the T concentration). The total amount of T present in each sample was estimated based on the T concentration and the volume of urine remaining. Of the 64 samples, 25 contained at least 2 μg of T per total volume. These 25 urines were assigned a new code and were treated as blind samples. They underwent a different sample preparation followed by analyses of the final fractions, containing the acetates of T, T precursors and T metabolites, by GC/MS (to verify identity and purity) and GC/C/IRMS (to measure the carbon isotope ratio). Pertinent characteristics of the urine samples under study, namely the subject number, weeks during which T was administered, sample collection weeks and T/E, which were all unknown during the GC/C/IRMS phase of the work, are reported in Table 1.

Steroid screen

The steroid screen included sample preparation by addition of [1,2-^{2}H]testosterone, column chromatography on XAD-2, enzymatic deconjugation with β-glucuronidase and sulfatase from *Helix pomatia*, formation of trimethylsilyl derivatives and GC/MS analysis in the selected-ion monitoring mode as described previously.[1] All urines were found negative for exogenous anabolic androgenic steroids. As expected, several of the urines had T/Es greater than six. T concentrations and total amounts of T were estimated from the screening data.

Reagents for GC/C/IRMS sample preparation

All solvents and reagents were of analytical grade. Sigma (Saint-Quentin Fallavier, France) provided T, cholesterol, dehydroepiandrosterone (DHEA), androsterone, etiocholanolone, 5-androstene-3β,17β-diol diacetate, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, 5β-androstane-3α,17β-diol, 5β-androstane-3β,17β-diol, and β-glucuronidase with sulfatase from *Helix pomatia*. Solid-phase extractions were carried out on Bakerbond Solid-Phase Extraction (SPE) columns filled with reversed-phase octadecyl (7020-07, J. T. Baker, Noisy-le-Sec, France) or adsorption phase silica gel (7086-03, J. T. Baker).

Steroid preparation for GC/C/IRMS analysis

The steroids were extracted, purified and acetylated as shown in Fig. 1 by modification of a previously described procedure.[13] Although TMS derivatives form faster than acetylated derivatives and have richer mass spectra, which is preferred for steroid identification purposes, silicon is not compatible with the combustion step of GC/C/IRMS , because it forms deposits on the copper wire, inactivates it and causes incomplete combustion. In addition, TMS derivatization adds three carbon atoms per derivatized site whereas acetylation adds only two and leads to a smaller modification of the carbon isotope ratio of the molecule of interest. We checked that acetylation caused no discrimination in the isotopic ratio measurement by treating T with different amounts of acetic anhydride and pyridine. Acety-

USADA1199

Table 1. Summary of T administration weeks, sample collection times, T/Es and carbon isotope ratios ($\delta^{13}C$) for precursors[a]

Subject	T admin. week	Sample collection week	T/E	Class	$\delta^{13}C$ (‰) C	P	Ta	M
1	13–18	1	1.6	N	−23.4	−23.8	−24.8	−25.1
		19	121	U	−21.3	—	−32.2	−31.7
2	13–18	13	3.3	N	−22.8	−23.8	−26.2	−24.8
		16	37	U	−23.6	−26.5	−31.4	−32.5
3	1–6	7	135	U	−24.7	−26.0	−30.4	−31.4
		13	4.9	N	−21.1	−24.8	−24.5	−26.6
4	1–6	1	1.6	N	−24.7	−27.2	−26.3	−25.7
		4	67	U	−24.9	−25.5	−29.7	−30.8
		13	2.2	N	−24.4	−26.2	−25.3	−26.2
5	1–6	1	1.6	N	−22.1	−25.2	−26.1	−25.5
		7	65	U	−23.2	−26.5	−29.9	−32.9
		16	1.8	N	−23.2	−25.7	−24.3	−23.9
6	13–18	10	1.8	N	−20.8	−27.0	−23.1	−27.1
		13	1.2	N	−24.6	−27.0	−23.5	—
		16	36	U	−22.4	−24.4	−31.0	−34.4
		19	72	U	−21.4	—	−29.4	−34.5
7	1–6	1	4.2	N	−23.3	−26.2	−25.2	−26.2
		4	32	U	−24.6	−27.3	−30.9	−32.4
		13	1.9	N	−25.4	−27.6	−22.2	−26.5
		16	1.5	N	−23.5	−27.5	−26.8	−29.2
8	1–6	1	1.0	N	−23.6	−25.9	−26.5	−27.3
		4	48	U	−21.7	−26.3	−30.2	−30.6
		7	76	U	−23.1	−26.9	−32.6	−33.2
		10	2.0	N	−22.6	−27.7	−27.1	−29.3
		16	0.8	N	−22.2	−27.7	−27.0	−27.6

[a] C = cholesterol acetate; P = 5-androstene-3β,17β-diol diacetate; Ta = testosterone acetate; metabolite M = 5α- and 5β-androstane-3α,17β-diol diacetate mixture. Subject, T/E and week data were unknown during $\delta^{13}C$ measurement. Dashes indicate that there was not sufficient material to measure $\delta^{13}C$. In addition, each sample is classified (CLASS) as T user (U if T/E > 6) or non-user (N if T/E < 6).

lation was carried out before, not after the high-performance liquid chromatographic (HPLC) separation, a change from the previously described procedure which produced an increased purity and yield of T. As a consequence of the modifications in the procedure, DHEA was no longer separated from androsterone and etiocholanolone, by either HPLC or GC, and therefore its carbon isotope ratio could not be measured. Instead, a different T precursor, namely 5-androstene-3β,17β-diol, could be purified and analyzed. In addition, 5β-androstane-3α,17β-diol diacetate was not separated from 5α-androstane-3α,17β-diol diacetate, so the measurement was done on the mixture of the two epimers.

Preparation of unconjugated cholesterol. Between 30 and 40 ml of urine were subjected to SPE on a C_{18} column (1 g of reversed-phase packing material for 25 ml of urine). The cholesterol fraction was eluted with chloroform–acetone (50:50, v/v; 2 × 6 ml). The column was retained for elution of one more fraction. The eluate was dried under nitrogen and the residue acetylated overnight at room temperature with acetic anhydride–pyridine (0.3 ml of each). After evaporation to dryness in a rotary vacuum evaporator (Speedvac, Bioblock Scientific, Illkirch, France), the acetylated extract was dissolved in dichloromethane–hexane (5:95, v/v; 10 ml), then applied to a silica gel SPE column. The cholesterol acetate fraction was eluted with dichloromethane–hexane (25:75, v/v; 6 ml). This solution was analyzed by GC/MS to check the compound identity and purity, then by GC/C/IRMS.

Preparation of deconjugated steroids. After elution of cholesterol from the above C_{18} column, the conjugated steroids were eluted with methanol (2 × 12 ml) and the eluate was evaporated to dryness. To hydrolyze the conjugated steroids, the methanolic residue was dissolved in 10 ml of 0.5 M acetate buffer (pH 5.5) and 0.50 ml of β-glucuronidase suspension was added as supplied; incubation was carried out at 55 °C for 3 h. After cooling, solid buffer (Na_2CO_3–$NaHCO_3$ (2:1, w/w), about 300 mg) was added. Liquid–liquid extraction was performed with diethyl ether (2 × 50 ml) and the solvent layers were combined and evaporated to dryness. The residue containing the hydrolyzed steroids was dissolved in ethyl acetate–hexane (5:95, v/v; 10 ml) and applied to a silica gel SPE column. The steroids were eluted with ethyl acetate–hexane (50:50, v/v; 6 ml). Acetylation of the purified extracts was carried out by

USADA1200

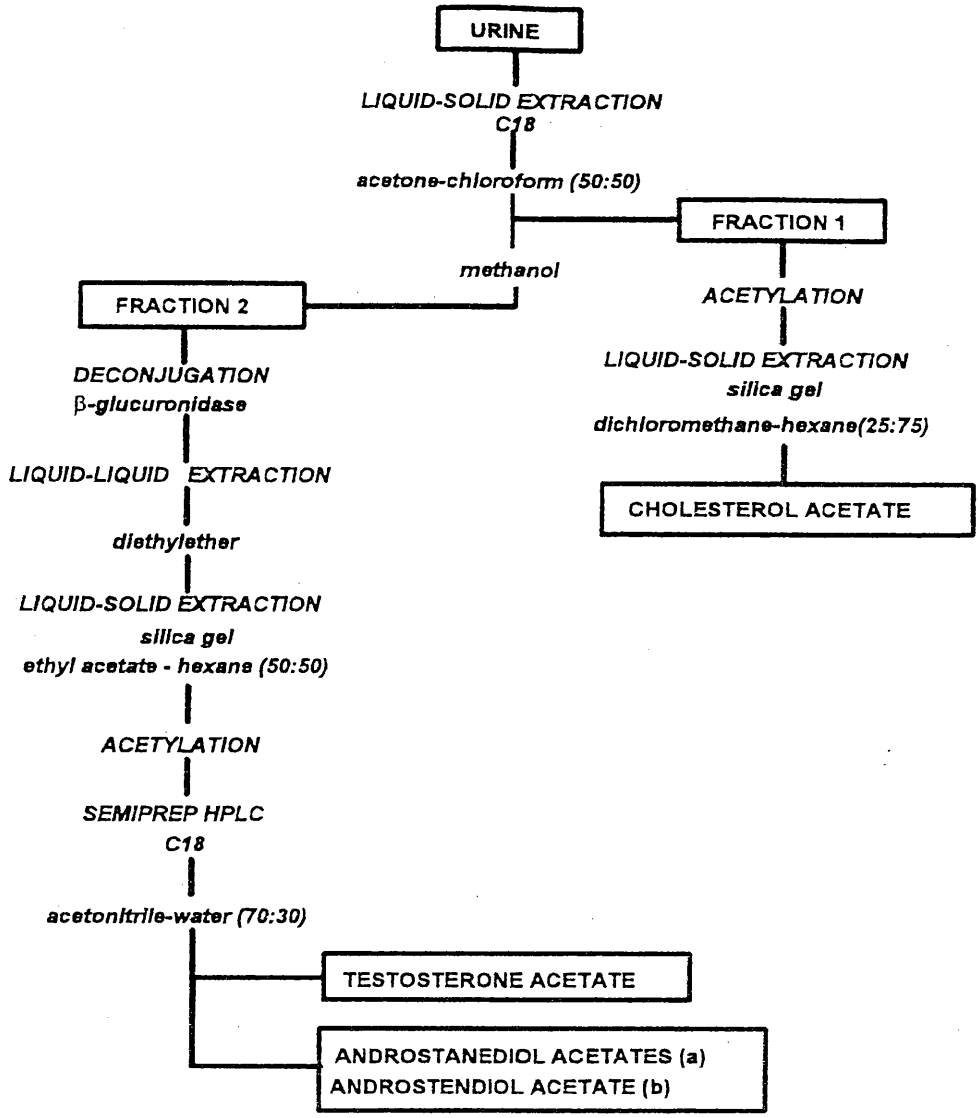


Figure 1. Flow chart of steroid preparation for GC/C/IRMS analysis. (a) 5α- and 5β-androstane-3α,17β-diol diacetates; (b) 5-androstene-3β,17β-diol diacetate.

overnight incubation at room temperature with a mixture of pyridine (1 ml) and acetic anhydride (0.8 ml). The reaction medium was evaporated to dryness in a rotary vacuum evaporator.

The dried residue from acetylation was dissolved in 50 µl of ethyl acetate and injected on to a semi-preparative C_{18} column: Spherisorb ODS2 (Interchim; 25 cm × 1 cm; 5 µm). The mobile phase, water–acetonitrile (30:70, v/v), was delivered by a Merck L-600 pump at a flow rate of 4 ml min^{-1}. The eluate was monitored by UV detection (Kratos Spectroflow 757 at 240 nm). T acetate was recovered in the retention time range 14.5–16 min (fraction 1). The mobile phase was changed to water–acetonitrile (20:80, v/v) 26 min after injection to allow faster elution of the androstanediol acetates and androstenediol acetate mixture in the retention time range 40–44 min (fraction 2). These fractions were analyzed by GC/MS to check compound identity and purity, then by GC/C/IRMS.

GC/MS analysis. GC/MS analyses of the acetylated derivatives were performed on a Hewlett-Packard (HP) Model 5890 gas chromatograph equipped with an HP Ultra-1 (100% methylsilicone) fused-silica capillary column (17 m × 0.2 mm i.d., 0.33 µm film thickness) linked to an HP 5970B mass-selective detector. The injections (1–2 µl) were made in the splitless mode using helium as carrier gas. For cholesterol acetate, the column oven temperature was increased from 200 to 300 °C at 15 °C min^{-1}. For the other fractions, the oven temperature started at 200 °C (held for 1 min), then was increased at 2 °C min^{-1} to 250 °C followed by 20 °C min^{-1} to 300 °C and maintained at the final temperature for 4 min.

Isotopic analysis

GC/C/IRMS analyses were performed on an HP Model 5890 gas chromatograph connected to a laboratory-made combustion furnace linked to a Finnigan MAT 252 isotope ratio mass spectrometer (Fig. 2). The gas chromatograph was equipped with an HP-1 column (Hewlett-Packard, 100% methylsilicone, 25 m × 0.32

USADA1201

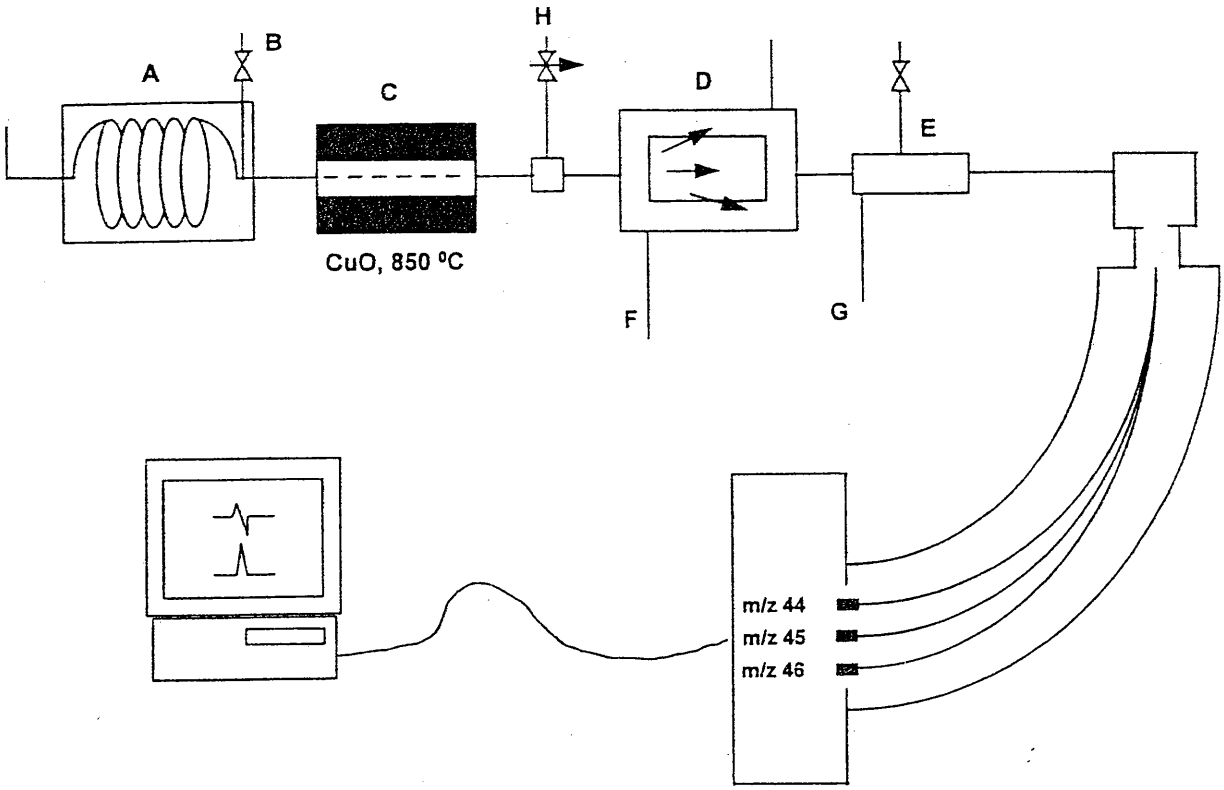


Figure 2. Schematic diagram of GC/C/IRMS instrument. A = gas chromatograph; B = solvent vent; C = combustion unit; D = water trap; E = open split; FG = helium inlets; H = oxygen inlet for CuO regeneration.

mm i.d., 0.52 μm film thickness). Helium was the carrier gas (20 psi). Samples were injected in the splitless mode at 285 °C. The GC separation began at 80 °C for 10 min, then the oven temperature was increased at 10 °C min^{-1} to 260 °C, followed by 5 ° min^{-1} to 315 °C, and maintained at the final temperature for 30 min. The temperature of the combustion oven was 800 °C and the oxidative catalyst was a wire of copper oxide (0.25 mm thickness in a quartz tube, 60 cm × 0.5 mm i.d.).

It is not the absolute $^{13}C/^{12}C$ isotope ratio, but rather the difference (δ) between the $^{13}C/^{12}C$ isotope ratio of the sample and that of an international standard that can be measured with great precision and accuracy. The international standard for carbon is a sample of carbonate obtained from the Pee Dee formation in South Carolina (Pee Dee belemnite or 'PDB') and relatively rich in ^{13}C. The δ standard notation for expressing carbon isotope ratios is defined as the relative difference in isotope ratio between the sample and PDB, calculated as

$$\delta\,^{13}C‰ = \left(\frac{R_{sample} - R_{PDB}}{R_{PDB}}\right) \times 10^3$$

where R_x refers to the $^{13}C/^{12}C$ of the sample or international standard and represented by $\delta\,^{13}C‰$ ('per mil'). When PDB became unavailable, the International Atomic Energy Agency (Vienna, Austria) calibrated a new standard against it: CO_2 gas (=international standard NBS 19). The definition of the $\delta\,^{13}C‰$ has not changed and continues to be the deviation from PDB. In the present work, NBS 19 was used to calibrate a tank of CO_2 reference gas. Pulses of the latter were introduced directly into the ion source during the backflush periods, which are designed to purge the injection solvent from the system with helium. Finally, the following alkanes were used between analyses as external standards: C_{16}, C_{18}, C_{20}, C_{22}, C_{24} and C_{28}.

The isotope abundance was calculated from the signals monitored simultaneously for the three major ions obtained from the isotopes of carbon and oxygen: *m/z* 44 for $^{12}C\,^{16}O\,^{16}O$, *m/z* 45 for ($^{13}C\,^{16}O\,^{16}O + {}^{12}C\,^{17}O\,^{16}O$) and *m/z* 46 for ($^{12}C\,^{18}O\,^{16}O + {}^{12}C\,^{17}O\,^{17}O$). After a correction[18] had eliminated the contribution of the $^{12}C\,^{17}O\,^{16}O$ ion from the *m/z* 45 signal, the *m/z* 45/44 ratio reflected that of $^{13}C/^{12}C$.

Each sample was submitted to two or three replicate measurements which were averaged. A typical standard deviation associated with these GC/C/IRMS determinations was 0.4‰. The limit of detection of the overall assay is 2 μg of testosterone per total volume of urine. In our experience, at least 100 ng of testosterone acetate must be injected into the GC/C/IRMS system in order to obtain a good signal-to-noise ratio.

Data analysis

All of the software used for data analysis was part of the Personal Computer–Statistical Analysis System (SAS Institute, Cary, NC, USA) version 6.08 for Windows. Multivariate analysis included principal component analysis, cluster analysis (using disjointed clustering and Euclidian distances computation) and discriminant analysis.

Principal components are new variables defined by mathematical combinations of the raw variables. The analysis[19] simplifies and improves data interpretation by reducing the number of variables and by creating non-correlated variables which reflect truly different 'dimensions' of the data. The first principal component is calculated to reflect as much variation as possible. Of the variation that is left unexplained by the first principal component, the second one must reflect as much as possible, and so on. The procedure defines as many principal components as there are raw variables and calculates what percentage of the variability of the data is accounted for by each principal component;[20,21] typically the last few principal components only account for a few per cent of the variability and may be ignored.

The cluster analysis looked for disjointed clustering and computed Euclidian distances.[22] We used the first three principal components and the 22 samples for which there was sufficient material for all variables to be measurable. We set the maximum number of clusters at two, since we were only interested in two groups, namely T users and non-users.

The discriminant analysis[23] tested the power of this approach to discriminate between T users and non-users. Of the 22 samples for which all variables were measurable, twelve were randomly selected to be the training set and the remaining ten were the test set. First, we labeled the five T users and seven non-users in the training set. Next, using the first three principal components as discriminating variables, the software computed discriminant functions on the training samples and classified each test sample as T user or non-user.

RESULTS AND DISCUSSION

In our previous work,[13] we found that the carbon isotope ratio was lower in exogenous T than in endogenous T. The value for endogenous substances may be affected by factors such as gender, ethnicity or diet. Indeed, the carbon isotope ratio of endogenous compounds reflects the proportions of C3 and C4 plant contributions to the diet; common C3 and C4 foods have values of -25.1 and $-10.8‰$, respectively,[24] so we expect baseline values to be within that range. Furthermore, $53.5 \pm 8.5\%$ of endogenous cholesterol, the precursor of endogenous T, is directly absorbed from the diet.[25] A typical North American diet, compared with a Western European diet, is expected to contain more C4 foods (including meat and dairy products from corn-fed animals), to have a higher input in ^{13}C and to result in higher baseline carbon isotope ratios.[26] In our experience so far, baseline values for T or C in European and North American subjects ranged from -24.5 to $-26.3‰$[13] and from -20.8 to $-26.8‰$, respectively, and the administration of different pharmaceutical Ts lowered the urinary T values. Therefore, we hope that the carbon isotope ratio for pharmaceutical Ts will always be outside the range of baseline values, even though we expect substantial variations within the latter. An early study of baseline serum cholesterol of South African residents reported remarkably stable values of $\delta^{13}C = -17‰$, which is expected to yield T with similar values,[15] and provides an example of a different baseline range in a different population. This makes it necessary to reference the measurement in the urine of interest to the natural isotopic ratio characteristic of that individual. The baseline value for the subject's own T when free of drug fulfils that need, but obtaining such urines under controlled conditions is impractical. To obtain intrinsic reference data from single urines, we reasoned that the isotopic ratio in T precursors should not be affected by exogenous T contribution, whereas T metabolites may reflect it. This is why, in addition to T acetate, we conducted isotopic ratio measurements on the acetates of T precursors cholesterol and 5-androstene-3β,17β-diol, and on those of T metabolites 5α-and 5β-androstane-3α,17β-diol.

The results of the isotopic measurements for the steroids in each urine appear in Table 1. For three urines, low volume and recovery precluded analysis for either 5-androstene-3β,17β-diol diacetate or the 5α-and 5β-androstane-3α,17β-diol diacetate mixture. We refer to the carbon isotopic measurement for cholesterol acetate as C, for 5-androstene-3β,17β-diol diacetate as P for precursor, for T acetate as Ta and for the 5α-and 5β-androstane-3α,17β-diol diacetate mixture as M for metabolites.

The carbon isotope ratio data on the 25 blind urines were obtained with no knowledge of the T/Es. Our goal was to compare the carbon isotope ratio approach with the $T/E > 6$ test as a means of identifying T users. Before the statistical analysis was performed, review of the raw data revealed that ten urines differed from the other 15 because both Ta and M were clearly decrea (Ta or $M \leqslant -30.0‰$). We tentatively classified these ten as T users and the other 15 as non-users, a conclusion which turned out to be in agreement with those of the cluster analysis, the discriminant analysis and the $T/E > 6$ test.

In the first step of the multivariate analysis, principal component analysis transformed the variables into new ones:

$$PRIN1 = 0.195211C + 0.227745P + 0.666504Ta + 0.682493M$$

$$PRIN2 = 0.678189C + 0.668824P - 0.251803Ta - 0.171259M$$

$$PRIN3 = 0.705791C - 0.693201P + 0.118116Ta - 0.085906M$$

$$PRIN4 = -0.061740C + 0.142404P + 0.691676Ta - 0.705332M$$

The first three principal components accounted for 48.9, 28.4 and 20.1%, respectively, of the variability of the data.

Cluster analysis defined two clusters. The first, second and third principal component means for the first cluster were -1.61, 0.33 and -0.20; for the second cluster they were 0.92, -0.19 and 0.11. Cluster 1 contained eight observations (36.36%) and cluster 2 contained the remaining 14 (63.64%). Looking up the

USADA1203

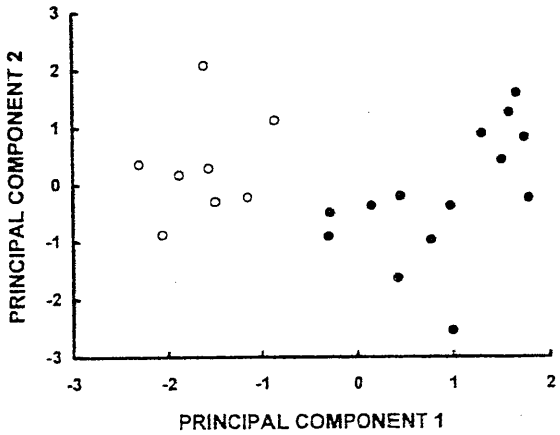


Figure 3. Cluster analysis results. The *x*- and *y*-axes are principal components 1 and 2, respectively. All urines in cluster 1 (○) were from T users (T/E > 6) and all urines in cluster 2 (●) were from non-users (T/E < 6).

corresponding T/Es showed that cluster 1 contained the eight T users (T/E > 6) and cluster 2 the 14 non-users (T/E < 6). Figure 3 shows the data points plotted against the first two principal components and highlights the two clusters.

Discriminant analysis tested the goodness of the separation on data in which three samples were T users and the rest were non-users. The discriminant functions for T users and non-users were DF = −8.87176 − 10.17479PRIN1 + 1.52167PRIN2 − 1.37050PRIN3 and DF = −5.37373 + 8.03788PRIN1 − 1.05549PRIN2 + 0.53145PRIN3, respectively. T users and non-users were correctly classified in their respective groups.

The carbon isotope ratio method appears comparable to the T/E > 6 test as a means of identification of T users with T/E > 30, but the high cost of the GC/C/IRMS instrument and the requirement for a large amount of sample preclude its use as a screening method. The most difficult aspect of the T/E test is that a very small number of males have been found with T/Es in the range 6–10 in the absence of T administration.[27–29] In such cases, the T/E > 6 criterion if applied to single urines would falsely classify these subjects as T users. For this reason, sport authorities do not act on the basis of a single urine with T/E > 6, instead they require additional tests;[5,12] healthy drug-free males are expected to have a relatively stable T/E.[30] We hope that the carbon isotope ratio test will be useful to discriminate between subjects with naturally elevated T/E and subjects having taken T.

Acknowledgements

We thank Isabelle Chanel for technical assistance and acknowledge support from the French Ministère de la Jeunesse et des Sports, the National Collegiate Athletic Association, the United States Olympic Committee and the National Football League.

REFERENCES

1. C. K. Hatton and D. H. Catlin, *Clin. Lab. Med.* 7, 655 (1987).
2. R. Massé, C. Ayotte and R. Dugal, *J. Chromatogr.* **489**, 23 (1989).
3. W. Schänzer and M. Donike, *Anal. Chim. Acta* **275**, 23 (1993).
4. *List of Doping Classes and Methods*. International Olympic Committee Medical Commission, Lausanne (1982).
5. *List of Doping Classes and Methods*. International Olympic Committee Medical Commission, Lausanne (1994).
6. M. Donike, K. R. Bärwald, K. Klostermann, W. Schänzer and J. Zimmerman, in *Sport: Leistung und Gesundheit*, edited by H. Heck, W. Hollman, H. Liesen and R. Rost, p. 293. Deutscher Arzte-Verlag, Cologne (1982).
7. L. Dehennin and A. M. Matsumoto, *J. Steroid Biochem. Mol. Biol.* **44**, 179 (1993).
8. R. V. Brooks, G. Jeremiah, W. A. Webb and M. Wheeler, *J. Steroid Biochem.* **11**, 913 (1979).
9. K. Carlstrom, E. Palonek, M. Garle, H. Oftebro, J. Stanghelle and I. Bjorkhem, *Clin. Chem.* **38**, 1779 (1992).
10. A. T. Kicman, H. Oftebro, C. Walker, N. Norman and D. A. Cowan, *Clin. Chem.* **39**, 1798 (1993).
11. L. Dehennin, *J. Endocrinol.* **142**, 353 (1994).
12. D. H. Catlin and D. A. Cowan, *Clin. Chem.* **38**, 1685 (1992).
13. M. Becchi, R. Aguilera, Y. Farizon, M.-M. Flament, H. Casabianca and P. James, *Rapid Commun. Mass Spectrom.* **8**, 304 (1994).
14. O. Breas, F. Fourel and G. J. Martin, *Analusis* **22**, 268 (1994).
15. J. T. Brenna, *Acc. Chem. Res.* **27**, 121 (1994).
16. P. Deines, in *Handbook of Environmental Isotope Geochemistry* edited by P. Fritz and J. C. Fontes, p. 329. Elsevier, New York (1980).
17. D. E. Matthews and J. M. Hayes, *Anal. Chem.* **50**, 1465 (1978).
18. H. Craig, *Geochim. Cosmochim. Acta* **12**, 133 (1957).
19. *SAS/STAT® User's Guide, Version 6*, 4th edn, Vol. 2, p. 1245. SAS Institute, Cary, NC (1989).
20. D. L. Massart, B. G. M. Vandeginste, S. N. Deming, Y. Michotte and L. Kaufman, *Chemometrics: a Textbook* p. 325. Elsevier, Amsterdam (1988).
21. B. F. J. Manly, *Multivariate Statistical Methods, a Primer* p. 59. Chapman and Hall, London (1986).
22. *SAS/STAT® User's Guide, Version 6*, 4th edn, Vol. 2, p. 823. SAS Institute, Cary, NC (1989).

23. *SAS/STAT® User's Guide, Version 6*, 4th edn, Vol. 2, p. 677. SAS Institute, Cary, NC (1989).
24. D. A. Schoeller, M. Minagawa, R. Slater and I.R. Kaplan, *Ecol. Food Nutr.* **18**, 159 (1986).
25. M. S. Bosner, R. E. Ostlund, Jr, O. Osofisan, J. Grosklos, C. Fritschle and L. G. Lange, *J. Lipid Res.* **34**, 1047 (1993).
26. D. Massicotte, F. Péronnet, C. Pitre, E. Adopo, G. R. Brisson and C. Hillaire-Marcel, *Eur. J. Appl. Physiol.* **67**, 402 (1993).
27. H. Oftebro, *Lancet* **339**, 941 (1992).
28. O. Namba, Y. Miyachi, M. Kawahara, M. Irie and Y. Kuroda, in *Hormones and Sport* edited by Z. Laron and A. D. Rogol, p. 275. Serono Symposia Publications, Raven Press, New York (1989).
29. E. Raynaud, M. Audran, J. F. Brun, C. Fedou, J. L. Chantal and A. Orsetti, *Lancet* **340**, 1468 (1992).
30. C. K. Hatton, D. H. Catlin, P. W. Straus and S. H. Starc presented at the 38th ASMS Conference on Mass Spectr etry and Allied Topics, Tucson, AZ (1990).

USADA1205

USADA1206

C. Ayotte, D. Goudreault, A. Lajeunesse, M. Cléroux, Y. Richard, A. Charlebois, J. -P. Couture and A. Fakirian

GC/C/IRMS and GC/MS in "Natural" Steroids Testing

Centre de recherche en santé humaine, INRS-Institut Armand-Frappier, Montréal

Introduction

Testing for the administration of natural steroids is a complex task requiring the identification and quantification of a number of parameters of the steroid profiles, one of which being the T/E[1] value. Complementary diagnostic information is gained from the determination of the urinary concentration of testosterone, epitestosterone, androsterone and etiocholanolone. By 1997, three different groups had proposed the GC/C/IRMS as a promising tool for the detection of the administration of testosterone (Aguilera et al., 1996; Becchi et al., 1994; Horning et al., 1997; Shackleton et al., 1997) and since then, others have described the values of the $^{13}C/^{12}C$ ($\delta\ ^{13}C\ ^{0}/_{00}$) of urinary androgens within the athletic populations and following the administration of DHT, epitestosterone or testosterone precursors such as DHEA.

In this paper, we present the results afforded by the analysis of urine samples collected following the administration of testosterone, androstenedione and DHEA as well as examples of the combined GC/MS and GC/C/IRMS individual "profiles" which have been proven to be useful in the investigation of putative doping cases, to discriminate inter alias, between the administration of a source of testosterone and 19-nortestosterone from a natural and systematic excretion of elevate T/E values or an increased endogenous norandrosterone production.

Experimental

"Androstenedione complex", DHEA and Nordione were purchased with an authorization from Health Canada (8572.090.98) from Price's Power International (International Nutrition and

[1] The T/E value is the corrected area ratio of both peaks.

Export, Newport News, Virginia 23608, USA). One capsule was administered to volunteers after verification of its content by GC/MS analysis. Urine samples were collected 24 hours before and up to one week after the administration and were prepared and analysed according to anabolic agents' procedure for the GC/MS steroid profiling. The preparation of the specimens for the GC/C/IRMS was carried out according to a modification of the method proposed by Shackleton (1997) for the analysis of testosterone and precursors. The underivatized extracts of hydrolysed steroids (Ayotte et al., 1996) were analysed as such when the concentration of norandrosterone was greater than 30 ng/mL or following an HPLC fractionation for lower levels. The identity of the peaks was verified by GC/MS. Authentic standards were purchased from Steraloids Inc. (Wilton, NH 03086).

Results and discussion

Testosterone, androstenedione and DHEA:

The administration of testosterone and precursors, androstenedione and DHEA can be detected by the GC/MS determination of parameters of the urinary steroid profile that are abnormal when compared to the ranges of values normally found in human (Donike et al., 1983, 1993). The oral intake of androstenedione and DHEA was shown to transiently increase the excreted T/E value in females and, although not systematically, in some males (Bowers, 1999; Bosy et al., 1998; Van Eenoo et al., 1998; Garle and Palonek, 1998, Uralets and Gillette, 1999; Lévesque and Ayotte, 1999; Lévesque et al., 1999). Other alterations of the urinary steroid profile, such as an abnormally high concentration of androsterone and etiocholanolone, the presence of the characteristic hydroxylated metabolites glucuro- and sulfoconjugated, 6α-hydroxyandrostenedione, 6β-hydroxyepiandrosterone for androstenedione and for DHEA, the increased excretion of 7β-hydroxydehydroepiandrosterone with suppression of 16α-hydroxyandrosterone, served as basis for reporting positive findings (Lévesque and Ayotte, 1999; Lévesque et al., 1999). The disruption of the normal urinary profiles of androgens metabolites can be demonstrated by comparison with the described population reference ranges that are summarised in table 1 (Ayotte, 1997 and reference cited therein; Lévesque and Ayotte, 1999; Catlin et al., 1997; Geyer et al., 1997; Baenzinger and Bowers, 1994). It also requires the

investigation of the athlete's previous or subsequent tests results in order to exclude the few individuals who naturally produce urine samples in which elevated T/E values are systematically measured. A systematic excretion of elevate T/E values reflects a normal condition, while a sudden increase deviating from the athlete's norm, is consequent with the administration of a source of testosterone, including the precursors (Ayotte, 1997; Geyer et al., 1997). The evaluation of some cases that we have made in the past years, based upon the variation of the individual's T/E values, are illustrated in Table 2.

Table 1: Description of the steroid profiles in the athletes' reference populations

Parameter	Description (97.5%)		Reference
	Male athletes	Female athletes	
T/E values	4.2 (n = 11000)	3.2 (n = 4667)	Lévesque and Ayotte, 1999
	5.2 (n = 5000)	6.3 (n = 1700)	Geyer et al., 1997
	6.5-7.0 (99%; n = 22806)		Baenzinger and Bowers, 1994
	5.6 (99%; n = 3700)		Catlin et al., 1997
T (ng/mL)	106 ng/mL (n = 9500)	26.4 ng/mL (n = 3740)	Lévesque and Ayotte, 1999
	137 ng/mL (n = 5000)	57 ng/mL (n = 1700)	Geyer et al., 1997
A (ng/mL)	6703 ng/mL (n = 9500)	5170 ng/mL (n = 4200)	Lévesque and Ayotte, 1999
	6689 ng/mL (n = 5000)	6439 ng/mL (n = 1700)	Geyer et al., 1997
Etio (ng/mL)	5294 ng/mL (n = 9500)	4938 ng/mL (n = 4200)	Lévesque and Ayotte, 1999
	4716 ng/mL (n = 5000)	6107 ng/mL (n = 1700)	Geyer et al., 1997

Table 2: Evaluation of elevate T/E values in athletes' samples

	T/E value			Remarks
Normally elevated cases				
	Mean	Variation	Range	
Male 1	4,8	23%	2,9 to 6,9 (n = 20)	
Male 2	1,3	13%	1,0 to 1,5 (n = 11)	
Male 3	5,8	25%	3,1 to 8,7 (n = 14)	
Female 1	0,8	38%	0,3 to 1,2 (n = 16)	
Female 2	1,1	43%	0,5 to 2,1 (n = 13)	
Female 3	6,1	39%	3,0 to 9.2 (n = 11)	
Positive cases				
Male 4	3,9	68%	2,3 to 10,6 (n = 8)	Positive test result: 10,6
	2,9	24%	2,2 to 3,2 (n = 7)	Excluding positive test result
Male 5	1,8	70%	0,9 to 7,3 (n = 21)	Positive test result: 7,3
	1,5	22%	0,9 to 2,2 (n = 20)	Excluding positive test result
Female 4	2,0	132%	0,6 to 11,6 (n = 16)	Positive test result: 11,6
	1,2	38%	0,6 to 1,9 (n = 14)	Excluding two elevate test results

USADA1207

USADA1208

Several groups have reported significantly changed carbon isotopic $^{13}C/^{12}C$ values (expressed as $\delta\ ^{13}C\ ^{0}/_{00}$) in the androgens metabolites excreted following the administration of testosterone (Aguilera et al., 1999, 2000, 2001), DHT, epitestosterone, DHEA (Shackleton et al., 1997; Ueki and Okano, 1999), and corticosteroids (Bourgogne et al., 2000). The methods described are based upon the comparison of the $\delta\ ^{13}C\ ^{0}/_{00}$ values of the urinary androgens metabolites to reference endogenous steroids, end products from earlier processes of the endogenous biosynthetic pathways. The urinary androgens metabolites most frequently analysed are androsterone, etiocholanolone, 5α- and 5β-androstane-3α,17β-diol, whereas the endogenous unaltered reference steroids are pregnanediol, pregnanetriol and cholesterol. The analysis of $\delta\ ^{13}C\ ^{0}/_{00}$ natural values amongst athletes did not indicate differences related to the nationality but to the diet (Aquilera et al., 1999, 2000, 2001; Shackleton et al., 1997, Ueki and Okano, 1999). To compensate for the different analytical methodologies and to allow for inter-laboratory comparison, it was proposed to use the ratio of the values of the androgens metabolites to those of the endogenous reference steroids in each sample (Shackleton et al., 1997).

19-nortestosterone and precursors, norandrostenedione and norandrostenediol:

The administration of 19-nortestosterone and of its precursors, 19-norandrostenedione and 19-norandrostenediol, results mainly in the excretion of 19-norandrosterone (NA) and 19-noretiocholanolone (NE), mostly found in the glucuroconjugated form. The period during which the metabolites can be detected, is drastically reduced when the oral preparations are taken and the relative amounts of NA and NE can vary based upon the individual and the product taken (Engel et al., 1958; Massé et al., 1985; Schänzer, 1996; Kintz et al., 1999). The low excretion of endogenous norandrosterone is normally not detected in human urine samples during routine doping control testing, with limits of detection of around 0.2 to 0.5 ng/mL, excepting specimens collected during pregnancy (Reznik et al., 1987). A more sensitive analytical technique such as the GC/HRMS, a larger volume of urine extracted and an extensive sample clean-up are needed to detect, identify and quantify endogenous NA, which in some male specimens was quantified at levels varying around 0.01 to 0.32 or 0.05 to 0.6 ng/mL, well below the limit of the IOC (Jeanneau et al., 1999; Le Bizec et al., 1999; Dehennin, 1999). In females, the level of NA is normally also very low, reaching a mean maximum value of 0.6 ng/mL during the ovulation

(Hemmersbach et al., 2000). The statistics collated by the IOC indicate that each year, around 0.24% of the A-samples analysed were reported to contain norandrosterone, making nortestosterone one of the anabolic agents most frequently found in athletes' samples along with testosterone.

Natural Steroids $\delta^{13}C\ ^{0}/_{00}$ values

We have measured the $\delta\ ^{13}C\ ^{0}/_{00}$ values of urinary steroids excreted in mixed athletes' samples of different nationalities and found them to be similar to those reported by others (table 3).

Table 3: Reference $\delta\ ^{13}C\ ^{0}/_{00}$ values measured in athletes' samples

		$\delta\ ^{13}C\ ^{0}/_{00}$		
Steroid	n	Mean	Std dev.	Range
Androsterone	78	-23,4	1,6	-19,9 to –26,6
Etiocholanolone	78	-23,3	1,3	-20,0 to –26,2
Pregnanediol	56	-24,9	1,2	-22,1 to –26,8
Pregnanetriol	68	-23,8	1,5	-20,8 to –26,8
Cholesterol	68	-24,2	1,5	-21,1 to –26,8

Synthetic $\delta^{13}C\ ^{0}/_{00}$ values

The GC/C/IRMS analysis of samples collected before and following the administration of testosterone and of a single oral dose of androstenedione and DHEA have shown that the ^{13}C content of the androgens metabolites differed significantly from their original value (stable within the course of the study) and from those of the reference steroids, going to the depleted values measured in synthetic steroids. Examples of results are summarised in tables 4, 5 and in figures 1, 2.

We are now using the combination of GC/MS and GC/C/IRMS to evaluate "testosterone" cases. Examples of normally and abnormally elevate T/E excretion are presented in table 6.

Table 4: $\delta\ ^{13}C\ ^{0}/_{00}$ values of urinary steroids modified by the administration of androstenedione.

Androstenedione			α-adiol	β-adiol	Etio	A	Pd	Pt	Chol.
Vol. 1	Before	Mean	-24,4	-24,3	-24,6	-24,3	-25,5	-23,8	-23,6
		Std dev	1,7	0,5	0,3	0,1	0,3	0,5	0,5
	After	Mean	-31,4	-32,9	-33,7	-31,7	-25,3	-24,1	-23,2
		Std dev	2,6	-3,2	1,9	2,4	0,5	0,8	0,8
	Difference		-7	-8,5	-9,1	-7,3	0,3	0,3	0,3
Vol. 2	Before	Mean	-[2]	-	-24,4	-23,8	-22,3	-23,0	-22,4
		Std dev	-	-	0,3	0,2	0,2	0,3	0,6
	After	Mean	-31,4	-32,4	-33,8	-33,9	-22,5	-23,7	-23,2
		Std dev	3,8	2,4	1,7	2,2	0,6	0,8	0,8
	Difference		-	-	-9,4	-10,1	-0,2	-0,6	-0,8

Table 5: $\delta\ ^{13}C\ ^{0}/_{00}$ values of urinary steroids modified by the administration of DHEA

DHEA			α-adiol	β-adiol	Etio	A	Pd	Pt	Chol.
Vol. 1	Before	Mean	-23,5	-25,0	-25,7	-25,8	-24,7	-24,4	-23,0
		Std dev	0,8	1	0,7	0,9	0,5	0,3	0,7
	After	Mean	-33,8	-31,8	-33,6	-34,0	-25,2	-24,5	-23,0
		Std dev	2,4	4,7	2,3	2,6	0,6	0,4	0,7
	Difference		-10,3	-6,8	-7,9	-8,3	0,5	0,1	0,1
Vol. 2	Before	Mean	-[2]	-23,3	-25,8	-24,8	-24,9	-23,9	-23,8
		Std dev	-	1,1	0,9	0,8	0,5	0,6	0,7
	After	Mean	-32,6	-33,7	-35,7	-34,1	-24,8	-24,5	-24,4
		Std dev	2,6	3,1	1,2	2,1	0,8	1,1	0,8
	Difference		-	-10,4	-9,9	-9,3	0,1	-0,6	-0,6

[2] The signal was too low to permit an accurate determination.

Table 6: Evaluation of testosterone cases using the combination of GC/MS and GC/C/IRMS

	$\delta\ ^{13}C\ ^{0}/_{00}$					
	T/E	Etio	A	Pd	Pt	A/Pd
Testosterone [3]	15,7	-28,5	-28,1	-23,8	-24,0	1,2
(Variation %)	(32%)	(2%)	(2%)	(3%)	(3%)	(3%)
Athlete 1 (negative)	4,2[4] (40%)	-22,6	-23,7	-23,7	-23,8	1,0
Athlete 2 (negative)	6,7[5]	-26,0	-26,3	-26,7	-26,2	1,0
	(23%)	(4%)	(3%)	(4%)	(0,3%)	
Athlete 3 (negative)	6,1[6]	-21,7	-22,2	-23,0	-23,5	1,0
	(25%)	(6,5%)	(3,8%)	(0,3%)	(6,1%)	
Athlete 4 (positive)	38	-31,3	-31,9	Cholesterol	-25,3	A/Cholesterol
				-25,7		1,2

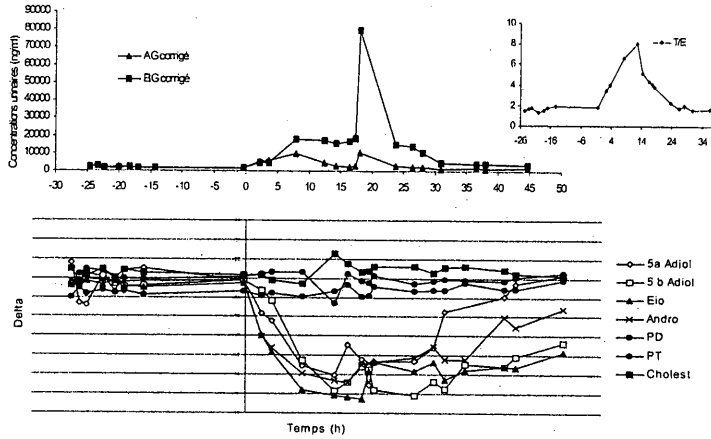


Figure 1: Variation of T/E values, corrected concentration of A and Etio and of $\delta\ ^{13}C\ ^{0}/_{00}$ values following the administration of a single dose of androstenedione in male volunteer 1.

[3] n = 15 urine samples (Medical treatment: testosterone enanthate once per week, two weeks collection)
[4] T/E : n = 8 $\delta\ ^{13}C\ ^{0}/_{00}$: single determination
[5] T/E : n = 4 $\delta\ ^{13}C\ ^{0}/_{00}$: n = 3
[6] T/E : n = 2 $\delta\ ^{13}C\ ^{0}/_{00}$: n = 2

USADA1209

USADA1210

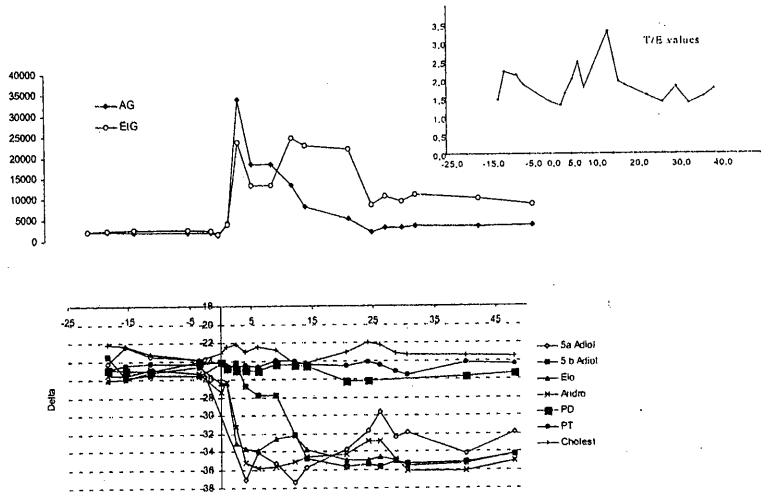


Figure 2: Variation of T/E values, corrected concentration of A and Etio and of δ ^{13}C $^{0}/_{00}$ values following the administration of a single dose of DHEA (200 mg) in male volunteer 3.

19-norsteroids

Twenty-five urine samples, analysed between 1992 to 1999, containing norandrosterone in amounts ranging from 35 to 11000 ng/mL, were re-analysed by GC/C/IRMS and so were the urine samples collected after the administration of norandrostenedione (commercial Nordione). The δ ^{13}C $^{0}/_{00}$ values measured in the excreted norandrosterone and noretiocholanolone were both significantly different than those of the reference steroids and of androsterone and etiocholanolone, excepting of course, athletes' samples in which elevate T/E values were also measured.

Endogenous norandrosterone (around 2 ng/mL), excreted during the 12th and 14th weeks of pregnancy of two different persons, was analysed by GC/C/IRMS following its isolation from a large volume of urine. The δ ^{13}C $^{0}/_{00}$ values then measured were found to be similar to the other endogenous steroids, androsterone, etiocholanolone, pregnanediol and pregnanetriol. The results are summarised in table 7.

Table 7: δ ^{13}C $^{0}/_{00}$ values of norandrosterone in 1) nortestosterone positive samples 2) norandrostenedione samples 3) pregnancy samples

	δ ^{13}C $^{0}/_{00}$ (mean values)					
	NA	NE	A	Pd	Pt	NA/Pd
Nortestosterone[7]	-31,1	-31,5		-23,0	-23,0	1,35
Norandrostenedione[8]	-31,6	-30,4	-22,7	-23,4	-22,4	1,36
Pregnancy 12th week[9]	-23,6	-	-24,9	-23,1		1,02
Pregnancy 14th week	-23,0	-	-26,1	-23,0		1,00

Conclusion

The combination of GC/MS and GC/C/IRMS has been proven useful to the evaluation of cases where the administration of "natural" steroids was suspected. The population reference ranges of the diagnostic parameters of both techniques are now well described. The ratio of the δ ^{13}C $^{0}/_{00}$ values measured in the steroids metabolites to the unaltered reference steroids is the only way to compensate for the many diets and for the different methods that are currently used in the laboratories. Alone, absolute values associated with either synthetic or natural steroids cannot form basis of the decision, significant differences having reported for the reference populations. The ratio of 1,1: 1,0 proposed by Shackleton (1997) for testosterone seems also adequate for the other steroids. The choice of the reference steroids must be made taking into account the alterations of the values that could be produced following the administration of precursors such as pregnenolone. When the concentration and the volume of specimen available permitted the injection of more than 20 ng, the ratios of the urinary synthetic norandrosterone to the unaltered reference steroids were always high since the contribution of natural norandrosterone can be virtually excluded. Natural values, similar to those of the reference steroids, were obtained during pregnancy.

[7] n = 25 urine samples
[8] Results of one excretion study. The mean value of commercial capsules: -32.7
[9] One determination: extracted from 560 mL and 380 mL respectively

Acknowledgements

The financial support of the Canadian Centre for Ethics in Sports (CCES), of the International Association of Athletic Federations (IAAF), the International Drug Testing and Management (IDTM) through their testing programmes is gratefully acknowledged. The skilled contribution of Alain Charlebois and Jean-Pierre Couture is invaluable. Jean-François Lévesque and André Lajeunesse received grants from the FCAR and from Sport Canada for which we are grateful.

Reference

Aguilera R., D.H. Catlin, M. Becchi, A. Phillips, C. Wang, R.S. Swerdloff, H.G. Pope and C.K. Hatton, J. Chromatogr.B., 727 (1999) 95

Aguilera R., M. Becchi, H. Casabianca, C.K. Hatton, D. H. Catlin, B. Starcevic and H.G. Pope, J. Mass Spectrom., 31 (1996) 169

Aguilera R., T.E. Chapman and D.H. Catlin, Rapid Commun. Mass Spectrom., 14 (2000) 2294

Aguilera R., T.E. Chapman, B. Starcevic, C.K. Hatton and D.H. Catlin, Clin. Chem., 47 (2001) 292

Ayotte C., D. Goudreault and A. Charlebois, J. Chromatogr., 687 (1996) 3

Ayotte C., Evaluation of elevated testosterone/epitestosterone values in athletes' urine samples, IAF New Studies in Athletics, 12:2-3 (1997) p. 87 and reference cited

Becchi M., R. Aguilera, Y. Farizon, M. M. Flament, H. Casabianca and P. James, Rapid Commun. Mass Spectrom., 8 (1994) 304

Bosy T.Z., K. A. Moore and A. Poklis, J Anal. Toxicol., 22 (1998) 455

Bourgogne E., V. Herrou, J.-C. Mathurin, M. Becchi and J. de Ceaurriz, Rapid Commun. Mass Spectrom., 14 (2000) 2343

Bowers L.D., Clin. Chem., 45 (1999) 295

Donike M., in Proceedings of the 10th Cologne Workshop on Dope Analysis, 7th to 12th June 1992, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth eds., Sport und Buch Strausse Ed. Sport, Koln, (1993) p. 47

Donike, M., K.-R. Barwald, K. Klostermann, W. Schanzer and J. Zimmermann, Nachweis von exogenem Testosteron in Sport: Leistung und Gesundheit, H. Heck, W. Hollmann, H. Liesenk, R. Rosts eds, Deutscher Arzte Verlag Koln, (1983) p.293

Engel L. L., J. Alexander and M. Wheeler, J. Biol. Chem., 231 (1958) 159

Garle M. and E. Palonek, Recent advances in doping analysis (6), Proceedings of the 16th Workshop on Dope Analysis, (1998) p. 181

Horning S., H. Geyer, M. Machnik, W. Schanzer, A. Hilkert and J. Oeßelmann, in Recent Advances in Doping Analysis (4), W. Schanzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke, eds, Sport und Buch Strauss Edition Sport, Koln (1997) p. 275

Kintz P., V. Crimele et B. Ludes, Acta Clin. Belg. Suppl., 1 (1999) 68

Lévesque J.-F. and C. Ayotte, in Recent Advances in Doping Analysis (7), Proceedings of the 17th Workshop on Dope analysis, (1999) p. 169

Lévesque J.-F. and C. Ayotte, idem, p. 213

Massé, R. C. Laliberté, L. Tremblay and R. Dugal, Biomed. Mass Spectrom., 12 (1985) 115

Reznik, Y., Herrou, M., Dehennin, L., Lemaire, M., and Leymaire, P., J. Clin. Endocrinol. Met., 64 (1987) 1086

Schanzer W., Clin. Chem., 42:7 (1996) 1001

Shackleton C.H.L., A. Phillips, T. Chang and Y. Li, Steroids, 62 (1997) 379

Shackleton C.H.L., E. Roitman, A. Phillips and T. Chang, Steroids, 62 (1997) 665

Ueki M. and M. Okano, Rapid Commun. Mass Spectrom., 13 (1999) 2237

Uralets V.P. and P.A. Gillette, J. Anal. Toxicol., 23 (1999) 357

USADA1211

USADA1212

U. Flenker and W. Schänzer

Kinetic Isotope Effects during Metabolism of Δ^4-Steroids

Institute of Biochemistry, German Sport University Cologne

Abstract

On principle kinetic isotope effects (KIEs) may occur under two conditions: At a rate limiting step and at a branchpoint of substance flux. The reduction of the double bond of Δ^4-steroids is the rate liniting step during metabolism of testosterone and related hormones. At the same time it represents a branchpoint, because two types of isomers occur. Isotopic fractionation therefore may be expected here. We could show that different δ^{13}C-values in fact can be measured for androsterone and eticholanolone, the most abundant representatives of each of the two branches. Different δ^{13}C-values as well could be observed between the 19-nor analogues of these compounds after administration of 19-norandrostendione to one volunteer.

1 Introduction

Kinetic isotope effects (KIEs), *i.e.* the fractionation of isotopes during reactions that are under kinetic control, fundamentally can be observed when

1. there is non-quantitative conversion of the educt(s) and
2. when different reaction constants exist for the different isotopes of the elements involved.

Lack of quantitative conversion occurs either at rate limiting steps or at branchpoints of the reaction. Due to the differing zero point energies, different reaction rates of isotopically substituted chemical bonds have to be assumed virtually all the time a bond is broken or formed [11, 12]. Whether this becomes manifest consequently depends on the fulfillment of the condition mentioned first.

The initial and rate limiting step during phase-1 metabolism of androgenous anabolic steroids is the reduction of the double bond between C-4 and C-5 [10]. This process constitutes a major branchpoint in steroid metabolism, because two classes of metabolites are formed (5α- and 5β-androstanes) and because the reaction appears to be irreversible under biological conditions. Two different enzymes are responsible for this, sited in separated compartments of the cell. As a bond between two carbons is affected, this step of steroid metabolism is a perfect candidate for fractionation of carbon isotopes to an detectable extent.

The most abundant metabolites resulting from this part of the metabolism of androgens (testosterone, androstendione, DHEA *etc.*) are androsterone (A) and etiocholanolone (E). A represents the 5α-, E the 5β-branch. We were interested in possible differences of the $^{13}C/^{12}C$-ratio between these compounds under normal biological conditions, *i.e.* in steady-state. At the same time we hypothesized that after administration of a synthetic Δ^4-steroid time dependent differences of the $^{13}C/^{12}C$-ratio could be observed between the two metabolic branches. We monitored the development of the $^{13}C/^{12}C$-ratio of 19-noretiocholanolone (NE) and 19-norandrosterone (NA) after oral application of a single bolus of 19-norandrostendione to one volunteer.

2 Methods

2.1 Analysis of E and A

Nine urine samples (U-1 – U-9) were analyzed after routine analysis had shown no evidence for presence of exogenous steroids. One quality control urine (QC) collected and pooled from eight males known to be free from synthetic steroids was analyzed as well. 5ml of urine were prepared.

Sample preparation on principle followed the method described in [7]. To achieve isolation of E and A before isotopic analysis a new HPLC cleanup procedure was developed:

- Solvents: H_2O/CH_3CN, starting at a ratio of 70:30 linearly changed to 45:55 within 17min.
- Flow: 1.8 ml/min.
- Column: Hewlett & Packard, ODS Hypersil, 250×4 mm, 5μm.

Several columns of similar specifications were tested. Only the model mentioned above yielded sufficient seperation of E and A.

After evaporation of the solvent the HPLC-fractions were taken up in 2-propanol. As the concentrations of E and A were known from routine steroid profiling, concentrations could be adjusted to $\approx$100μg/ml uniformly in all resulting samples.

Isotopic measurements were performed by gas chromatography / combustion / isotope ratio mass spectrometry (GC/C/IRMS). General setup of the whole device was as described in [7]. The chromatographic column was a M&N Optima-δ3 (Macherey & Nagel, Düren, Germany), 17m length, 0.25mm inner diameter, 0.25μm film thickness. Injections (1μl) were performed in splitless mode at a headpressure of 30psi. Temperature was isothermal at 60 °C for 90s, raised to 246 °C at 30K/min and then raised to 278 °C at 3K/min. Finally the temperature was raised to 290 °C at 30K/min and was kept constant for 5min.

Five replicate measurements were performed on each HPLC-fraction. Data were analyzed by means of ANOVA.

Table 1: Two factor ANOVA for five replicate measurements of the $\delta^{13}C$-values of androsterone and etiocholanolone (factor 'Ster.') nested within ten urine samples (factor 'Spl.'). Factor 'Spl.' as well as its interaction with factor 'Ster.' shows highly significant influence.

	Df	SSQ	MSQ	F	p
Spl.	9	20.71	2.30	18.60	< 0.0001
Spl.×Ster.	10	24.61	2.46	19.89	< 0.0001
Resid.	80	9.90	0.12		

Table 2: Linear model for the $\delta^{13}C$-values of 19-noretiocholanolone (NE) and 19-norandroterone (NA) depending on time after application of 100mg 19-norandrostendione. Intercepts show significant difference. Slopes are siginificantly different, where the slope for NA is not significantly different from zero.

	$\delta^{13}C$	SE	p		$\frac{d\delta^{13}C}{dt}$	SE	p
NE	-30.25	0.24	< 0.0001	NE	0.045	0.015	< 0.01
NA	-28.83	0.24	< 0.0001	NA	-0.024	0.015	≈ 0.12

2.1.1 Analysis of 19-norsteroids

One volunteer (male, 49y, 76kg) orally administered 100mg of 19-norandrostendione. Urine samples were collected 10, 16.2 and 20 hours after application. Samples were treated as described above. NA and NE were isolated by HPLC and underwent isotopic analysis in different runs. Again five replicate measurements were performed.

Data were analyzed by iterative fitting of linear models with 'time' being the independent variable.

3 Results

Table 1 shows the results of a two factor ANOVA for the $\delta^{13}C$-values of A and E (factor 'Ster.') nested within the ten analyzed urine samples (factor 'Spl.'). Factor 'Spl.' and the interaction of 'Spl.' and 'Ster.' are highly significant. A graphical representation of the corresponding data is given in figure 1. Data are presented as $\bar{x} \pm s$ of $\delta^{13}C$-values of E and A for each urine sample *vs.* the grand mean. Figure 1 also contains asterisks indicating significant differences ($p \leq 0.05$) in $\delta^{13}C$-values between E and A within sample after BONFERRONI-correction. In nine out of ten samples the average $\delta^{13}C$-values of E is lower than that of A. Out of these nine cases seven are significant.

Table 2 shows the parameters of the linear model describing the dependence of the ^{13}C-enrichment of NE and NA on time after application of 19-norandrostendione. Model selection techniques (iterative analysis of variance) showed that different intercepts and different slopes are indeed necessary to describe the data. No evidence for curvature was found in the data. A graphical representation of the model is given in figure 2.

USADA1213

USADA1214

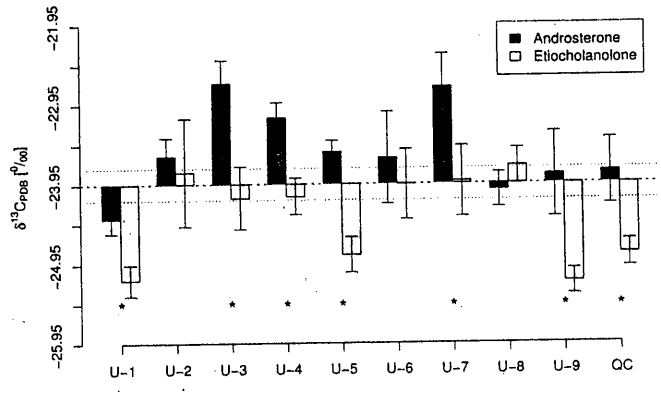


Figure 1: δ^{13}C-values of androsterone (A) and etiocholanolone (E) from nine routine urine samples (U-1 – U-9) and from one urine collected and pooled from eight males (QC). Values are represented as $\bar{x} \pm s$ relative to grand mean. Dotted lines indicate 99%-confidence limits of the grand mean. Asterisks indicate significant difference between E and A within sample after BONFERRONI-correction.

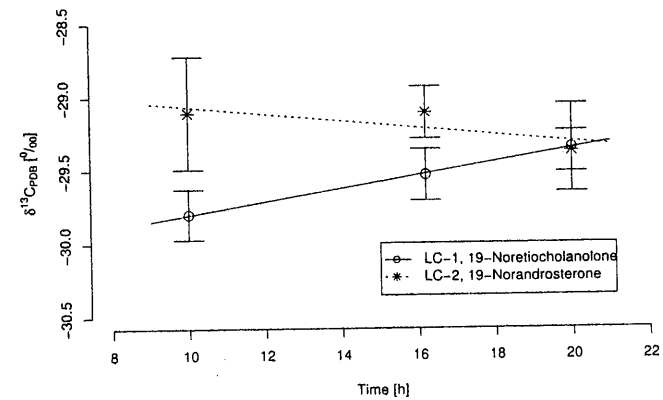


Figure 2: δ^{13}C-values of 19-norandosterone and 19-noretiocholanolone after administration of 100mg 19-norandrostendione. Symbols and error bars show $\bar{x} \pm s$. Fitted lines represent the linear model described in table 2.

4 Discussion

The isotopic data for E and A clearly indicate a fractionation of the stable carbon isotopes ^{13}C and ^{12}C during the reduction of the double bond between C-4 and C-5. E is depleted in ^{13}C relative to A. The degree of fractionation appears to differ from individual to individual. Isotopic fractionation theoretically depends on

1. isotopic fractionation during the reaction itself (probably different in 5α- and 5β-reduction),
2. the substrate concentration and
3. the magnitude of the fluxes of metabolites through both pathways.

Hence from the present data a definite model cannot be formulated. Nevertheless our observations are in good accordance with theory: Given that a depletion of ^{13}C occurs during 5β-reduction the residual and therefore the 5α-metabolites must be isotopically enriched. Biological variation of the activities of any of the two relevant enzymes will cause different fluxes through the metabolic pathways, which easily could explain the significant interindividual variation observed in differences between δ^{13}C-values of E and A.

5α- and 5β-androstanes are frequently analyzed with respect to their ^{13}C/^{12}C-ratios in doping control to detect abuse of synthetic steroids in sport [1–7, 9]. Published results are somewhat contradictory concerning possible isotope fractionation however. In a recent study Aguilera *et al.* found that acetylated 5β-androstane-3α, 17β-diol was isotopically slightly enriched *vs.* acetylated 5α-androstane-3α, 17β-diol [5]. In an earlier investigation Aguilera *et al.* found no significant difference in isotopic composition between A and E [4]. Again acetylated compounds were used for GC/C/IRMS analysis. Flenker *et al.* reported ^{13}C-depleted E relative to A using underivatized material [7]. Aguilera *et al.* presented similar data based on acetylated androstanediols once more [3].

One possible reason for the inconsistent results might be that GC/C/IRMS is sensitive towards lack of chromatographic baseline seperation. Depending on the chromatographic conditions 5α- and 5β-isomers of androstanes are difficult to separate. Goodman and Brenna [8] report an apparent enrichment of around 2 ‰ for the first of two coeluting compounds (methyltridecanoate and butylated hydroxytoluene) at an overlap of 10 % valley. Increasing overlap worsens the situation dramatically. Former work of our group [7] likewise showed that in contrast to isolated measurements of the same laboratory standards E apparently is ^{13}C-depleted *vs.* A when the two compounds are measured together. Chromatographic seperation was better than 10 % valley.

Unfortunately the reported isotopic fractionations are in an order of magnitude that allows for explanation by true KIEs as well as by systematic analytical error. In the present study we circumwent possible problems caused by lack of chromatographic separation by introduction of HPLC isolation of both compounds of interest.

The data obtained from our execretion study with 19-norandrostendione generally confirm the results. Obviously 5β-metabolites of androgens are depleted in ^{13}C *vs.* their 5α-counterparts. This supports our hypothesis of a KIE present during 5β-reduction. As the

substrate concentration decreases the isotopic fractionation decreases as well. No evidence was found for a KIE during 5α-reduction.

However theoretically two approximately parallel curves should be obtained for the two branches with or without isotope effect present in 5α-metabolism. Our results suggest different slopes for ^{13}C in NE and NA over time. It has to be emphasized that the slope for NA is not significantly different from zero. A possible explanation might be a distribution of the substrate into different compartments **before** the Δ^4-reduction. It is known that 5α- and 5β-reductase indeed are sited in the endoplasmatic reticulum and in the cytoplasm of hepatocytes respectively [10].

References

[1] R. Aguilera, M. Becchi, H. Casabianca, C. M. Hatton, D. H. Catlin, B. Starcevic, and H. G. Pope Jr. Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J Mass Spec*, 31:169–176, 1996.

[2] R. Aguilera, M. Becchi, C. Grenot, H. Casabianca, and C. K. Hatton. Detection of testosterone misuse: comparison of two chromatographic sample preparation methods for gas chromatographic-combustion/isotope ratio mass spectrometric analysis. *J Chromatography B*, 687:43 – 53, 1996.

[3] R. Aguilera, D. H. Catlin, M. Becchi, A. Phillips, C. Wang, R. S. Swerdloff, H. G. Pope, and C. K. Hatton. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. *J Chromatography B*, 727:95-105, 1999.

[4] R. Aguilera, T. E. Chapman, and D. H. Catlin. A rapid screening assay for measuring urinary androsterone and etiocholanolone $\delta^{13}C$ ($^0/_{00}$) values by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Comm Mass Spec*, 14:2294–2299, 2000.

[5] R. Aguilera, T. E. Chapman, B. Starcevic, C. H. Hatton, and D. H. Catlin. Performance characteristics of a carbon isotope ratio method for detecting doping with testosterone based on urine diols: Controls and athletes with elevated testosterone/epitestosterone ratios. *Clin Chem*, 47(2):292–300, 2001.

[6] M. Becchi, R. Aguilera, Y. Farizon, M.-M. Flament, H. Casabianca, and P. James. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rapid Comm Mass Spec*, 8:304–308, 1994.

[7] U. Flenker, S. Horning, E. Nolteernsting, H. Geyer, and W. Schänzer. Measurement of $^{13}C/^{12}C$-ratios to confirm misuse of endogenous steroids. In W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, editors, *Proceedings of the Manfred Donike Workshop*, number 6 in Recent advances in doping analysis, pages 243–256. Sport und Buch Strauß, Köln, 1999.

[8] K. J. Goodman and J. T. Brenna. Curve fitting for restoration of accuracy for overlapping peaks in gas chromatography/combustion isotope ratio mass spectrometry. *Anal Chem*, 66:1294–1301, 1994.

[9] S. Horning, H. Geyer, M. Machnik, W. Schänzer, A. Hilkert, and J. Oeßelmann. Detection of exogenous testosterone by $^{13}C/^{12}C$-analysis. In W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, editors, *14th Cologne Workshop on Dope Analysis*, number 4 in Recent Advances in Doping Analysis, pages 275–283. Sport und Buch Strauß, Köln, 1996.

[10] W. Schänzer. Metabolism of anabolic androgenic steroids. *Clinical Chemistry*, 42(7):1001–1020, 1996.

[11] D. A. Schoeller. Isotope fractionation: Why aren't we what we eat? *Journal of Archaeological Science*, 26:667–673, 1999.

[12] H. Simon. *Biophysik*, chapter 7, pages 235 – 249. Springer-Verlag, Berlin, Heidelberg, New York, 1977.

USADA1215

USADA1216

Richard K.R. Taylor[1,2], Adam T. Cawley[1,2], Rymantas Kazlauskas[1], Graham J. Trout[1] & Adrian V. George[2]

Validity of carbon isotope ratio measurements for decomposed urine samples

1. Australian Sports Drug Testing Laboratory (ASDTL), National Measurement Institute (NMI), Sydney, Australia
2. School of Chemistry, University of Sydney, Sydney, Australia

Introduction

Unintentional deconjugation of steroids in urine samples sent to WADA accredited laboratories for analysis is an indication of microbiological action. Criteria used to subsequently detect this decomposition in urine samples include changes in pH, a percentage of free testosterone greater than 5 as measured by Gas Chromatography-Mass Spectrometry (GC-MS) and the presence of 5a and 5b-androstane-3,17-diones, [1,2]. These decomposed samples no longer have steroid profile values that can be representative of the originally collected urine due to steroid interconversions and metabolism by microorganisms. It has been proposed that carbon isotope ratio ($\delta^{13}C$ (‰)) measurements made by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) on the urinary residual androsterone (A) and etiocholanolone (Et) may alleviate issues associated with the accurate measurement of the testosterone to epitestosterone (T/E) ratio conducted on samples that would be deemed to be decomposed by doping control laboratories [2].

This problem will arise when samples that present GC-MS screening T/E values of greater than 6 but GC-MS confirmation of these samples reveal that a significant amount of testosterone (T) is detected in the unconjugated (free) form. This presents an interesting scenario when GC-C-IRMS analysis of these samples reveals ^{13}C depleted T metabolite values that would indicate administration of an endogenous steroid (Table 1 shows results for 2 such cases).

Table 1: Cases of decomposed urine samples with high T/E values

	CASE 1	CASE 2
T/E	8.2	4.0
Free T (%)	24.6	11.2
δ^{13}C Et (‰)	-28.5	-28.3
δ^{13}C A (‰)	-27.2	-27.3
δ^{13}C ERC (‰)	-23.1	-21.4
Difference (‰)	4.8	6.4

Experimental

Urine was collected, with informed consent, from 15 volunteers (5 female, 10 male) aged between 20 and 45 years of age. Separate aliquots (3 mL) of these urine samples were stored for 1, 2 and 3 months at -10 and 22.4 °C. Measurements of pH and specific gravity were made prior to the initial analysis and each monthly analysis.

T/E determinations were made following the routine T confirmation procedure: addition of d_3T (4 µg/mL, 50 µL) as surrogate, isolation of urinary steroid conjugates using Serva PADII resin, reconstitution of the dried conjugate fraction with phosphate buffer pH 7 (0.2 M, 1.5 mL), extraction with ether to give the free fraction, enzyme hydrolysis of the aqueous layer using β-glucuronidase from *E.coli* K12 (EC 3.2.1.31, 50 µL) at 50 °C for 1.5 hours, hexane extraction of the hydrolysate at pH 10, addition of 17α-methylT (4 µg/mL, 50 µL) as internal standard, then derivatisation of the free and conjugated fractions to form TMS enol-ethers (MSTFA/TMSI, 55 µL). Measurements were made using an Agilent 6890/5973 GCMS using a HP1 column (17m, 0.11um film, 0.2mm diameter) using routine screening conditions.

GC-C-IRMS analysis was carried out on the residual androsterone, etiocholanolone and 11-ketoetiocholanolone (internal marker) using the conditions described previously [4] on steroid extracts obtained following the addition of 5α-androstan-3β-ol (100 µg/mL, 50 µL) as surrogate and the addition of phosphate buffer pH 7 (0.2 M, 1.5 mL) before enzyme hydrolysis as described above. Solid phase extraction of the hydrolysed urinary steroid metabolites was

performed using Varian BondElut ® Certify cartridges that were conditioned with methanol (3 mL), water (3 mL), loaded with the sample hydrolysate, washed with water (3 mL), water/methanol (90:10, 3 mL), hexane (1 mL), then eluted with ethyl acetate/methanol (95:5, 3 mL). 17α-methylT (100 µg/mL, 50 µL) was added as internal standard.

Results and discussion

Changes in pH values.

The pH of the urines changed during the storage at room temperature to basic pH values indicating formation of ammonia. The range of change was 0.3 to 3.4 pH units.

Correlation of δ^{13}C values and free T

No urine sample displayed significant variation (>1‰) between δ^{13}C values of Et and A measured initially and those obtained following storage. No sample was found to have δ^{13}C values of Et or A that were ^{13}C depleted (i.e. more negative) than the proposed threshold of -27.0‰ [3]. The % free T for all samples varied significantly (up to 68%) as time progressed. Consequently, no correlation was observed between changes in δ^{13}C values and % free T (Figure 1).

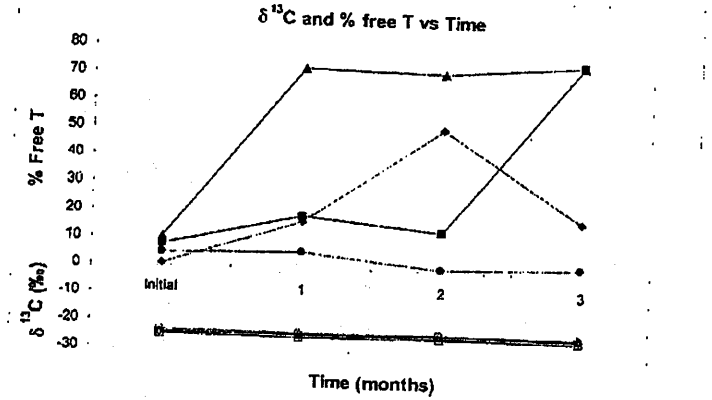


Figure 1: Selected samples showing no correlated change between δ^{13}C and % free T values

USADA1217

USADA1218

Analysis of decomposition products

The $\delta^{13}C$ values of 5β-androstanedione and 5α-androstanedione were determined for samples that contained sufficient amounts of each. No difference could be established between the $\delta^{13}C$ values of Et and A with their respective C-3 keto analogues. The results from samples 10 and 12 are shown in Table 2 as an example.

Table 2: $\delta^{13}C$ values of Et and A and their respective decomposition products

	SAMPLE 10	SAMPLE 12
$\delta^{13}C$ Et (‰)	-23.6	-22.9
$\delta^{13}C$ 5β-dione (‰)	-24.0	-22.3
$\delta^{13}C$ A (‰)	-23.3	-21.9
$\delta^{13}C$ 5α-dione (‰)	-23.8	-20.3

Conclusion

Fractionation (i.e. discrimination of ^{13}C) of the isotopic distribution due to enzymatic processes was not observed from microbiological degradation of urinary steroid metabolites. These results demonstrate the possibility of $\delta^{13}C$ measurements being valid for urine samples deemed to be decomposed based current criteria of % free T or androstanediones.

References

1. C. Ayotte, A. Charlebois, S. Lapointe, D. Barriault, M. Sylvestre. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (4), SPORT und BUCH Strauß, Cologne, 1997, pp. 127-137.
2. R. de la Torre, X. de la Torre, J. Segura, M.T. Smeyers, R. Ventura, J.M. Torres, C. Alia, T. Baró. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (6), SPORT und BUCH Strauß, Cologne, 1999, pp. 223-235.
3. G.J Trout, J.H. Rogerson, A.T. Cawley, C.W. Alma. *Aust. J. Chem.* 56:175-180.
4. A.T. Cawley, J.H. Rogerson, K. Rahman, G.J. Trout, R. Kazlauskas. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (11), SPORT und BUCH Strauß, Cologne, 2003, pp. 183-193.

CSIRO PUBLISHING

Current Chemistry

www.publish.csiro.au/journals/ajc

Aust. J. Chem. **2003**, *56*, 175–180

Developments in Sports Drug Testing

Graham J. Trout,[A,E] *Jill H. Rogerson*,[A,B] *Adam T. Cawley*[A,C] *and Christopher W. Alma*[A,D]

[A]Australian Sports Drug Testing Laboratory (ASDTL), Australian Government Analytical Laboratories, Pymble 2073, Australia.
[B]Now at Research and Development, Australian Government Analytical Laboratories, Pymble 2073, Australia.
[C]School of Chemistry, University of Sydney, Sydney 2006, Australia.
[D]Department of Health Sciences, University of Technology, Sydney 2007, Australia.
[E] Author to whom correspondence should be addressed (e-mail: graham.trout@agal.gov.au).

Manuscript received: 4 November 2002.
Final version: 21 January 2003.

Introduction

Sport is a major part of our lives, both as passive entertainment and for active participation. Sporting prowess is admired and, in many sports, financially rewarded. The desire to be the best has led many athletes to obtain an unfair advantage over their rivals by the use of performance-enhancing drugs. One of the major ways to deter such cheating is by carrying out drug testing on urine samples collected both in and out of competition. The systematic testing of athletes for performance enhancing drugs began in 1968 with testing for stimulants such as amphetamine,[1] which were then in common use in some sports. Now there are 27 laboratories around the world accredited by the International Olympic Committee (IOC) who regularly test samples from athletes for a range of banned drugs.

The IOC has, in their anti-doping code,[2] a list of banned substances and methods; included are anabolic steroids, stimulants, narcotics, diuretics, and peptide hormones. The first four categories consist of compounds of relatively low molecular weight that are amenable to analysis by techniques such as gas chromatography (GC) or gas chromatography–mass spectrometry (GC-MS), which are common to most laboratories analyzing organic chemicals. Thus GC-MS has been the backbone of sports drug testing for the last decade with most IOC laboratories having a number of GC-MS methods, each one of which detects a class of compounds such as steroids. When new steroids or stimulants have been developed it has been relatively simple and inexpensive to determine their metabolites and add these compounds to one of the existing screens.

However there are two groups of compounds that are not amenable to analysis by GC-MS. The first group comprises the anabolic endogenous steroids, which are present naturally in the body such as testosterone and androstenedione. Whilst these endogenous steroids are quite separable by GC, conventional MS analysis can only confirm their identity but not establish their origin, whether the bottle or the body. Synthetic and naturally occurring testosterone have small differences in the ratio of carbon-12 to carbon-13 present in their molecules with the synthetic material being ^{13}C-deficient. The specialized technique of carbon isotope ratio mass spectrometry (IRMS) is capable of measuring these differences. The second group of compounds comprises the peptide hormones and other large biomolecules, which cannot be analysed by GC and which need techniques such as liquid chromatography–mass spectrometry (LC-MS) for their analysis and identification. This paper will describe two areas where new technologies are being used for drug testing in sport, the first being the detection of endogenous steroid abuse and the second the detection of compounds which are capable of enhancing the oxygen-carrying capacity of the blood. Endogenous steroids are used because they are anabolic in nature and can increase muscle mass. Thus they are most likely to be used in power sports such as weightlifting and sprinting. Compounds which can enhance the oxygen-carrying capacity of the blood, such as erythropoietin (EPO) and haemoglobin-based blood substitutes, are most likely to be used in endurance events such as road cycling and the marathon.

Endogenous Steroid Detection and Carbon IRMS

Stable carbon isotopic composition of a substance is generally measured by isotope ratio mass spectrometry. IRMS systems derive isotope ratios by monitoring simple gases, which, in the case of carbon, is CO_2. Accordingly m/z 44, 45, and 46 are monitored, corresponding to isotopomers $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$, and $^{12}C^{16}O^{18}O$. This type of mass spectrometer utilizes Faraday cups positioned in the ion beam with each cup collecting ions of a particular mass to charge ratio.

Carbon isotope ratios (CIRs) are expressed in terms of $\delta^{13}C$ values, with units in per mille (‰), Equation (*1*).[3]

$$\delta^{13}C = \left(\frac{r_{\text{sample}}}{r_{\text{standard}}} - 1\right) \times 1000 \qquad (1)$$

Measurements are expressed on a scale where the zero point is set by the isotopic composition of Pee Dee Belemnite, a marine carbonate that is relatively enriched in ^{13}C. This results in many $\delta^{13}C$ values being negative. Results are

© CSIRO 2003
10.1071/CH02227 0004-9425/03/020175

expressed in per mille because the differences are generally very small; a difference of 1‰ on the $\delta^{13}C$ per mille scale corresponds to a change in 0.001099 atom-% in natural ^{13}C abundance.[4]

Carbon IRMS analysis requires samples to be converted to carbon dioxide prior to entering the MS source. Historically this is achieved via an off-line oxidation. Analysis by IRMS coupled to a gas chromatograph (GC-C-IRMS) analysis has necessitated the development of capillary-scale combustion techniques, allowing components which are eluted from the GC column to be oxidized on-line prior to travelling in a gas stream to the IRMS source. GC-C-IRMS instruments must also include a means of removing water from the gas stream. Water can protonate CO_2 producing $^{12}C^{16}O^{16}O^{1}H^{+}$, which also has a mass-to-charge ratio of 45, and can therefore interfere with the final result.

All living matter is derived through photosynthesis from CO_2 in the atmosphere, where $\delta^{13}C = -7‰$, or in marine environments, where $\delta^{13}C = 0‰$.[5] Plants with C-3 and C-4 metabolisms discriminate to different degrees against $^{13}CO_2$, and the isotope ratios in biochemicals from plants reflect the biochemical grouping of the plants.[6] The carbon isotopic composition in humans reflects that in the diet. This is why human breath samples of Americans, who eat a lot of corn and corn products (C-4 plants), are depleted in ^{13}C compared with Europeans, whose diet is more wheat-based (C-3 plants).[5]

Isotopic composition is useful in a sports drug-testing context because synthetic steroids are derived from stigmasterol obtained from soy,[7] a C-3 plant which gives synthetic testosterone $\delta^{13}C$ values in the range −25.9 to −32.8‰.[8] Synthetic copies of other endogenous steroids are generally more depleted in ^{13}C, and can have $\delta^{13}C$ values as low as −35.6‰.[9] The basis for gonadal testosterone is the precursor molecules consumed in the variety of vegetables in the human diet, or in the diet of the animal protein providers, and in Australia falls in the range −19 to −25‰.[10] The difference in carbon isotope ratio between the gonadal and synthetic steroids, although small, is statistically significant and measurable. Administration of synthetic endogenous steroids leads to a measurable depletion of ^{13}C content in the parent compound present in the urine[9] and also in its metabolites.[11,12]

Ketosteroid metabolites including androsterone (A) and aetiocholanolone (E) are often the most concentrated of the urinary steroids and can be analyzed easily without derivatization.

As mentioned earlier, diet can affect the $\delta^{13}C$ values of human steroids and their metabolites. Diets can vary greatly with culture and sport, and this is accounted for in IRMS doping analysis by measuring the isotopic composition of a 'background marker' steroid that is not involved in the androgenic anabolic steroid pathway. 11-Ketoaetiocholanolone (11-ketoE), part of the corticosteroid pathway,[13] is a suitable background marker. Its concentration in the urine is on-scale with A and E. Work in the ASDTL has found the $\delta^{13}C$ of 11-ketoE to range between −18.5 and −24‰.

Having analysed a set of androgenic steroid metabolites and at least one background marker (as recommendeded by the IRMS working group of the IOC Doping Control Laboratories[14]), the analyst then must decide if the data shows evidence of doping. The most common method is to use a ratio of the androgen results to those of the background marker, with values greater than a threshold[11] being indicative of steroid misuse. Other approaches used in ASDTL include taking the difference between the androgen and background markers and using the average of the androgen results in the calculations.

A DHEA Administration Study

A recent study conducted at ASDTL, in collaboration with the School of Chemistry of the University of Sydney, investigated the effect that oral dehydroepiandrosterone (DHEA) administration had on the carbon isotope ratio of urinary steroid metabolites. DHEA is an endogenous androgen that is rumoured to be administered by athletes with the aim of increasing levels of the more active androgens such as testosterone and dihydrotestosterone.[15] The IOC prohibits DHEA administration in athletes,[2] however the detection of administered DHEA has been difficult due to an incomplete understanding of DHEA metabolism as well as inter-individual variations.[16]

GC-C-IRMS demonstrated that a single oral administration of DHEA (100 mg) to a male subject lowered the ^{13}C content of E and A relative to 11-ketoE. E and A are inactive metabolic products of the androgen biosynthetic pathway, of which DHEA is the primary precursor.[17] The administration study was provided by Robert Weatherby, Head of the Exercise Science and Sport Management School at Southern Cross University (Lismore, Australia). It included the informed consent of the healthy 30-year-old male volunteer and approval of the Human Ethics Committee of the University. A total urine sample was collected at the time of the single oral DHEA (100 mg) administration (time zero) and 13 total urine samples collected in a 52-hour period post-administration. The $\delta^{13}C$ values obtained from each sample are shown below in Figure 1.

The minimum $\delta^{13}C$ values observed for E and A were −32.1 and −30.0‰ respectively at 9 h post-administration. The magnitude of ^{13}C depletion of E following DHEA administration was interesting, since the $\delta^{13}C$ value of DHEA in the preparation administered to the subject was determined to be −31.3‰. Prior to this study it was assumed that the

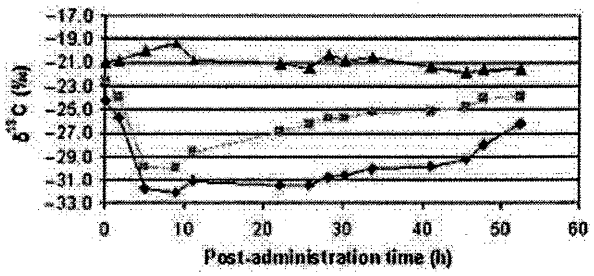


Fig. 1. $\delta^{13}C$ trends of E (◆), A (■), and 11-ketoE (▲) of a male subject following a single DHEA administration.

USADA1220

minimum $\delta^{13}C$ values of E and A following DHEA administration would be ^{13}C-enriched (less negative) in relation to the administered DHEA due to the dilution effect of E and A from an endogenous origin. In this study at least, the dilution effect of endogenous E was not as pronounced as expected. This has further emphasised the lack of understanding regarding the metabolism of administered DHEA to form E. These GC-C-IRMS results complement the findings of previous studies involving GC-MS analysis of urine extracts obtained after DHEA administration that show prolonged post-administration increases in E concentration.[16,18,19]

As expected, DHEA administration had no significant effect on the $\delta^{13}C$ value of 11-ketoE, which had an average of −20.9‰ and a range of 2.6‰ thought to be the result of dietary changes. Importantly however, alteration in $\delta^{13}C$ values of E and A in relation to each other and with respect to 11-ketoE did provide evidence of DHEA administration. Repeatability in these GC-C-IRMS measurements was verified by determination of $\delta^{13}C$ of 17α-methyltestosterone (17-meT) internal standard. The average $\delta^{13}C$ value of the 17-meT was −32.9‰ with a standard deviation of 0.4‰.

The detection period of administered DHEA in this subject can be determined by examining the return of $\delta^{13}C$ E and $\delta^{13}C$ A to the baseline values that were observed at the time of administration. This is also thought to be dependent on the dilution effect of E and A from endogenous origin. The results of this study showed that DHEA administration in this subject could be confirmed by GC-C-IRMS analysis for up to 11 h post-administration. This was based on the three concurrent criteria used by ASDTL to distinguish endogenous steroid metabolites from those of synthetic origin:

- $\delta^{13}C$ E and $\delta^{13}C$ A < −27.0‰
- Averaged difference (Equation (*2*)) >4.00‰
- Averaged ratio (Equation (*3*)) >1.15

$$\left(\frac{\delta^{13}C\,E + \delta^{13}C\,A}{2}\right) - \delta^{13}C\,11\text{-ketoE} > 4.00‰ \quad (2)$$

$$\left(\frac{\delta^{13}C\,E + \delta^{13}C\,A}{2}\right) \Big/ \delta^{13}C\,11\text{-ketoE} > 1.15 \quad (3)$$

The data obtained were used to calculate the maximum values of (*2*) and (*3*) which were 11.7‰ and 1.60 respectively at 9 h post-administration. Regarding the detection period, at 11 h post-administration $\delta^{13}C$ E and $\delta^{13}C$ A had values of −31.1 and −28.6‰, respectively, while (*2*) and (*3*) had values of 9.0‰ and 1.43 respectively. Accounting for the prolonged effect that DHEA administration had on $\delta^{13}C$ E, it may have been possible to confirm endogenous steroid abuse for up to 48 h post-administration. At this time, $\delta^{13}C$ E had a value of −28.0‰ while (*2*) and (*3*) had values of 4.4‰ and 1.20 respectively.

It is important to consider that while the current criteria applied to the results of this study would confirm endogenous steroid administration, it may not specifically identify DHEA abuse. In future, doping control laboratories will aim to identify specific androgen administration using GC-C-IRMS analysis. To achieve this, a greater understanding of androgen metabolism is required, particularly in relation to the effects of administration. Future research will hopefully establish metabolic markers of specific androgen abuse and investigate statistical methods of analysis for the confirmation of that androgen.

The Detection of Compounds Which Enhance the Oxygen Carrying Capacity of the Blood

There is a range of ways of enhancing the normal oxygen carrying capacity of one's blood. These include blood transfusions (either autologous or homologous), the injection of EPO to stimulate new red blood cell production, and the infusion of haemoglobin-based blood substitutes. This paper will only consider the latter two methods. The detection of EPO abuse is especially difficult when compared to the detection of other endogenous substances, such as anabolic steroids, because not only is EPO a complex, heavily glycosylated protein with a molecular weight in excess of 30 kDa but also it is present in very low concentrations in both blood and urine. This problem is compounded by the complex glycosylation of EPO, which gives rise to multiple isoforms with no one molecular species being more than a few percent of the total mass. The detection and measurement of EPO in a typical blood sample is not within the capability of current, routine LC-MS technology, and so other techniques such as immunoassay detection must be used. However as EPO occurs naturally, its mere presence even at elevated levels is not proof of doping. Two approaches have been developed for the detection of doping with EPO, directly using urine and indirectly using blood. The urine test does not use conventional drug-testing techniques but relies on electrophoresis to separate the isoforms of EPO on a pH gradient gel.[20] The procedure uses a patented double-blotting technique to detect the very low concentrations of EPO in the presence of high concentrations of other proteins.[21] The method works because the current forms of recombinant EPO have slightly different isoforms to those found in normal urinary EPO. The indirect method for the detection of EPO relies on the fact that several whole blood and serum parameters are significantly altered when EPO is administered. EPO does not in itself enhance the oxygen-carrying capacity of the blood but rather stimulates the production of new red blood cells by the bone marrow. The changes in blood chemistry and cell morphology which accompany this accelerated erythropoiesis are the basis of the indirect blood test.[22]

Blood substitutes are being developed by several companies as an alternative to blood transfusions because of the problems in obtaining sufficient whole blood for medical demands coupled with the difficulty in ensuring that the blood is not contaminated. The products being developed are designed to be used in operations and emergencies in place of donated blood.[23] They do not, and are not intended to, carry out all the functions of whole blood but only make up volume expansion after blood loss and deliver oxygen to the tissues. A haemoglobin-based blood substitute has been shown to have a higher oxygen-carrying capacity per unit volume than whole blood,[24] hence infusing a subject of normal blood

USADA1221

capacity with blood substitutes should increase their oxygen-carrying capacity. It is thus highly likely that some athletes involved in endurance sports will seek to use blood substitutes to improve their performance. The drug currently used for such cheating is erythropoeitin, however EPO has the disadvantage that it is slow acting—taking at least two weeks to produce the desired effect—whilst the effect of a blood substitute would be virtually instantaneous.

This work only deals with the detection of haemoglobin based blood substitutes often referred to as haemoglobin-based oxygen carriers or HBOCs.

The HBOCs that have progressed to clinical trials are all based on some form of haemoglobin, either human or bovine. The haemoglobins are all chemically modified in some way to prevent the toxic effects that result from the presence of high concentrations of free haeomoglobin in plasma.[25] In normal blood, effectively all the haemoglobin is within the red blood cells in its native tetramer structure (two alpha and two beta subunits) and very little is free in the plasma. If injected into the plasma, haemoglobin dissociates into dimers and monomers. In HBOCs the chemical modifications involve some form of crosslinking of the alpha and beta subunits of haemoglobin as well as polymerization or conjugation to another large molecule. These modifications are designed to markedly increase the molecular weight of the haemoglobin of the HBOC, so that it can function effectively and safely as an oxygen carrier outside a red blood cell.

The detection of blood substitutes requires that blood be collected as so far there are no means to detect their presence in urine. The tests were developed using Hemopure (kindly supplied by the Biopure Corporation, Cambridge, MA) but were designed so that they would work with any HBOC based on polymerized haemoglobin. Hemopure is a gluteraldehyde-polymerized bovine haemoglobin and is the only HBOC so far approved for use in humans. Firstly a screening test is needed which can eliminate most negative samples whilst detecting all or most of the suspicious samples. The screening test should be fast and inexpensive. Secondly a confirmation test is required which can correctly identify any blood substitute based on polymerized haemoglobin. This test must be able to produce evidence that can withstand legal challenge. The cost can be relatively high, as an effective screen will mean that very few samples will need to be tested by this method.

The use of any HBOC will raise the concentration of haemoglobin in the plasma. Thus the measurement of the haemoglobin content of plasma (Hb) will be an effective indicator of HBOC use. However haemolysis of red blood cells could also produce an elevated plasma Hb. Fortunately the number of samples containing a significant level of haemolysis is very small. When the plasma Hb concentration was determined on 45 samples from elite athletes, the highest plasma Hb concentration found was $2\,g\,L^{-1}$ and that in only one subject. For an HBOC to give a significant performance advantage the amount infused would need to increase the total amount of haemoglobin present by at least 5%. As all this additional haemoglobin would be in the plasma this equates to a plasma Hb concentration of approximately $14\,g\,L^{-1}$. Thus a screen based on confirming any sample with a plasma

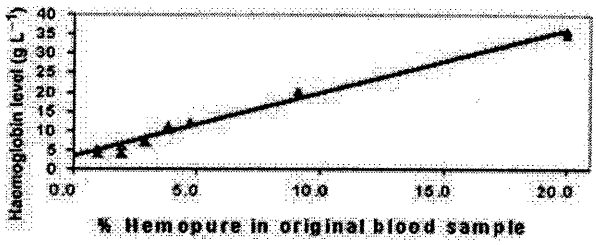


Fig. 2. Plasma haemoglobin levels resulting from the addition of Hemopure to blood.

Hb concentration above $2\,g\,L^{-1}$ would detect all those using HBOCs with a very low rate of false positives. Figure 2 shows the concentration of haemoglobin in plasma found when varying amounts of Hemopure were spiked into blood.

Whilst it is highly unlikely that the haemolyis of red blood cells could produce haemoglobin levels approaching those corresponding to HBOC use, it is necessary to prove that the free haemoglobin is from an extraneous source. As the most-developed HBOCs are based on polymerized haemoglobin, methods have been chosen which have the potential to distinguish natural haemoglobin with a molecular weight of 64 kDa or below from polymerized haemoglobin with a molecular weight of 128 kDa and above. Two methods have been used to confirm that the free haemoglobin is from an HBOC, one based on size-exclusion high performance liquid chromatography (SEC-HPLC) with diode array detection[26] and the other based on peptide mapping by electrospray mass spectrometry of tryptic digests of haemoglobin.[27]

When a sample of Hemopure is analysed by means of SEC-HPLC, it is possible to resolve the polymeric material on the basis of its molecular weight and polymerized haemoglobins can be readily separated from native haemoglobin. Figure 3 shows the excellent separation between the polymerized haemoglobin in Hemopure and native human haemoglobin.

As expected, human haemoglobin elutes as a single peak later than the multiple peaks corresponding to the various polymeric haemoglobins present in Hemopure. The UV/visible spectra of all the haemoglobins are essentially the same. When Hemopure was spiked into plasma it was again possible to detect the polymeric haemoglobins and distinguish them from native haemoglobin arising from haemolysis of the red blood cells. The peaks corresponding to polymeric haemoglobin were still detectable at a Hemopure concentration of $2\,g\,L^{-1}$, which means that any use of an HBOC that was detected by the screening procedure would be able to be confirmed.

Whilst the detection of peaks having the spectrum of haemoglobin at retention times corresponding to molecular weights significantly above 64 kDa is clear proof of the presence of a chemically modified haemoglobin, it does not specifically identify which particular HBOC has been used. This is not essential to prove doping but would be useful additional information. The technique of peptide mapping using electrospray mass spectrometry was used in an attempt to show that the peptide fragments detected after enzymatic digestion with trypsin were different in native

USADA1222

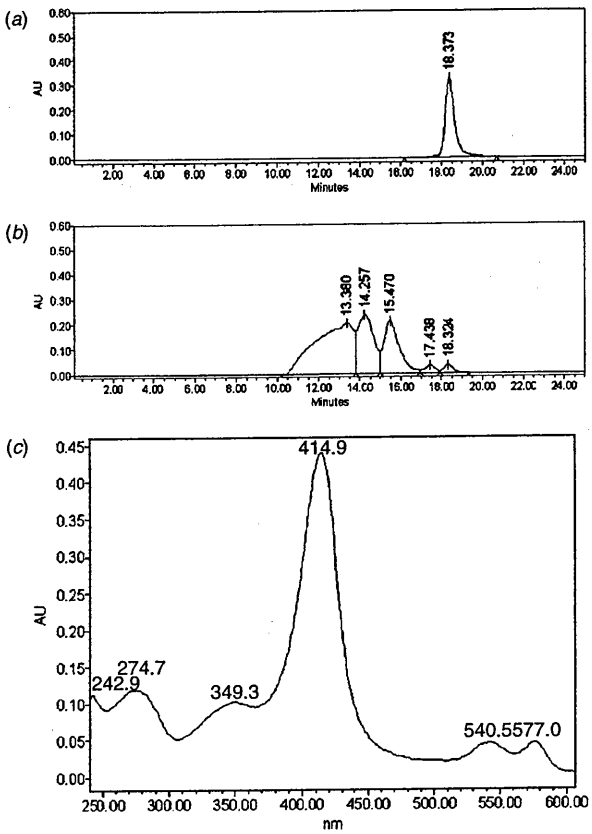


Fig. 3. Size-exclusion chromatograms of (*a*) human haemoglobin and (*b*) Hemopure. (*c*) UV/vis spectrum of eluted haemoglobin.

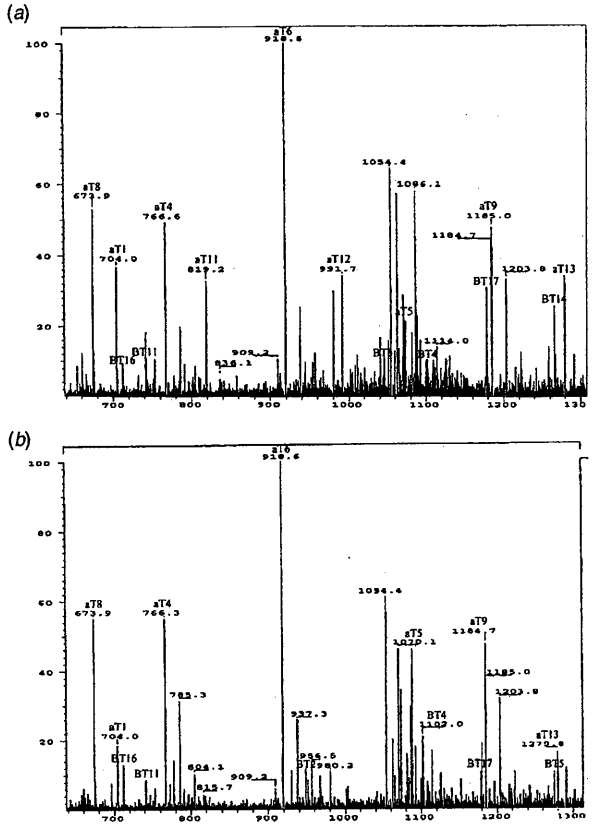


Fig. 4. Mass spectra obtained from tryptic digests of (*a*) bovine haemoglobin and (*b*) Hemopure.

haemoglobin than in polymerised haemoglobin. Hemopure and bovine haemoglobin were digested with trypsin and the resulting digests directly infused into a high-resolution mass spectrometer (MAT 900, Finnigan, Bremen) equipped with an atmospheric pressure ionization interface (API III, Varian, Finnigan). The spectra obtained are shown in Figure 4.

The spectra obtained are similar as expected but with two obvious differences. The peaks at *m/z* 819 and 992 are missing from the Hemopure sample. These peaks arise from the tryptic peptides from amino acid positions 93–99 and 100–127. This indicates that in Hemopure the lysine at position 99 in the alpha chain is not being cleaved. This is consistent with the glutaraldehyde cross-linking occurring between the alpha chains at position 99. Thus the mass spectral results demonstrate both that Hemopure contains haemoglobin of bovine origin and that it has been chemically modified. Further work is needed to determine whether similar results can be obtained with Hemopure that is present in plasma. The mass spectral analysis of peptide digests has the potential to identify the type of haemoglobin used and the chemical modifications made with other HBOCs.

Conclusions

The application of new analytical methodologies such as IRMS and LC-MS to drug testing in sport has the potential to further deter the use of banned substances and methods. However as the doping substances become more complex or more similar to endogenous compounds then the methods which must be applied become more complex and expensive. In the case of HBOCs it should be possible to have detection methods in place before HBOCs are available for general use. However post-competition blood samples must be available for the methods to be applied. At present only urine samples are collected and hence this is a further example of the additional cost and complexity that is and will continue to be associated with the fight against drugs in sport.

Acknowledgments

We thank the Australian Government's 'Backing Australia's Sporting Ability' initiative and the World Anti-Doping Agency for funding major aspects of this work.

References

[1] *Minutes of the 67th Session of the International Olympic Committee*, Mexico **1968** (IOC: Lausanne), pp. 12–13, A11.
[2] See: http://multimedia.olympic.org/pdf/en_report_21.pdf.
[3] H. Craig, *Geochim. Cosmochim. Acta* **1957**, *12*, 133.
[4] W. Brand, *J. Mass Spectrometry* **1996**, *31*, 225.
[5] W. Meier-Augenstein, *LC-GC*, **1997**, 17.
[6] B. N. Smith, S. Epstein, *Plant Physiol.* **1971**, *47*, 380.
[7] J. J. W. Coppen, *Trop. Sci.* **1979**, *21*, 125.

USADA1223

[8] X. de la Torre, J. C. Gonzalez, S. Pichini, J. A. Pascual, *J. Pharmaceutical Biomed. Anal.* **2001**, *24*, 645.
[9] M. Ueki, M. Okano, *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2237.
[10] J. Rogerson, G. Trout, R. Kazlauskas, unpublished results; to be presented at the 21st Cologne Workshop on Dope Analysis, Cologne, **2003**.
[11] C. H. L. Shackleton, A. Phillips, T. Chang, Y. Li, *Steroids* **1997**, *62*, 379.
[12] C. H. L. Shackleton, E. Roitman, A. Phillips, T. Chang, *Steroids* **1997**, *62*, 665.
[13] C. H. L. Shackleton. *Clin. Chem.* **1983**, *29*, 246.
[14] W. Schanzer, C. Ayotte, R. Kazlauskas, X. de la Torre, M. Ueki, Presentation at the 20th Cologne Workshop on Dope Analysis, Cologne, **2002**.
[15] See: http://www.mja.com.au/public/issues/171_4_160899/corrigan/corrigan.html. Verified 3 February 2003.
[16] L. Dehennin, M. Ferry, P. Lafarge, G. Pérès, J. P. Lafarge, *Steroids*, **1998**, *63*, 80.
[17] H. L. J. Makin, D. B. Gower, D. N. Kirk, *Steroid Analysis* **1995** pp. 268–272 (Blackie: London).
[18] V. P. Uralets, P. A. Gillette. *J. Anal. Toxicology* **2000**, *24*, 188.
[19] A. Cawley, *Honours thesis* **2001**, pp. 17–18 (The University of Sydney: Sydney).
[20] F. Lasne, J. de Ceaurriz, *Nature* **2000**, *405*, 635.
[21] F. Lasne, *J. Immunol. Methods* **2001**, *253*, 125.
[22] R. Parisotto, M. Wu, M. J. Ashenden, K. R. Emslie, C. J. Gore, C. Howe, R. Kazlauskas, K. Sharpe, G. J. Trout, M. Xie, A. G. Hahn. *Haematologica* **2001**, *86*, 128.
[23] T. M. S. Chang, *Trends Biotechnol.* **1999**, *17*, 61.
[24] G. S. Hughes Jr., E. P. Yancey, R. Albrecht, P. K. Locker, S. F. Francom, E. P. Orringer, E. J. Antal, E. E. Jacobs Jr., *Clin. Pharm. Ther.* **1995**, *58*, 434.
[25] H. F. Bunn, *Transfus. Clin. Biol.* **1995**, *6*, 433.
[26] G. S. Hughes Jr., E. J. Antal, P. K. Locker, S. F. Francom, W. J. Adams, E. E. Jacobs Jr., *Crit. Care Med.* **1996**, *24*, 756.
[27] T. Yang, D. R. Horejsh, K. J. Mahan, E. J. Zaluzec, T. J. Watson, D. A. Gage, *Anal. Biochem.* **1996**, *242*, 55.

USADA1224

Clinical Chemistry 47:2
292–300 (2001)

Drug Monitoring and Toxicology

Performance Characteristics of a Carbon Isotope Ratio Method for Detecting Doping with Testosterone Based on Urine Diols: Controls and Athletes with Elevated Testosterone/Epitestosterone Ratios

RODRIGO AGUILERA,[1*] THOMAS E. CHAPMAN,[1] BORISLAV STARCEVIC,[1] CAROLINE K. HATTON,[1] and DON H. CATLIN[1,2]

Background: **Carbon isotope ratio methods are used in doping control to determine whether urinary steroids are endogenous or pharmaceutical.**

Methods: **Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) was used to determine the $\delta^{13}C$ values for 5β-androstane-3α,17β-diyl diacetate (5βA), 5α-androstane-3α,17β-diyl diacetate (5αA), and 5β-pregnane-3α,20α-diyl diacetate (5βP) in a control group of 73 healthy males and 6 athletes with testosterone/epitestosterone ratios (T/E) >6.**

Results: **The within-assay precision SDs for 5βA, 5αA, and 5βP were ± 0.27‰, ± 0.38‰, and ± 0.28‰, respectively. The between-assay precision SDs ranged from ± 0.40‰ to ± 0.52‰. The system suitability and batch acceptance scheme is based on SDs. For the control group, the mean $\delta^{13}C$ (SD) values were −25.69‰ (± 0.92‰), −26.35‰ (± 0.68‰), and −24.26‰ (± 0.70‰), for 5βA, 5αA, and 5βP, respectively. 5βP was greater than 5βA and 5αA ($P < 0.01$), and 5βA was greater than 5αA ($P < 0.01$). The means − 3 SD were −28.46‰, −28.39‰, and −26.37‰ for 5βA, 5αA, and 5βP, respectively. The maximum difference between 5βP and 5βA was 3.2‰, and the maximum 5βA/5βP was 1.13. Three athletes with chronically elevated T/Es had $\delta^{13}C$ values consistent with testosterone administration and three did not.**

Conclusions: **This GC-C-IRMS assay of urine diols has low within- and between-assay SDs; therefore, analysis of one urine sample suffices for doping control. The means, SDs, ±3 SDs, and ranges of $\delta^{13}C$ values in a control group are established. In comparison, testosterone users have low 5βA and 5αA, large differences between 5βA or 5αA and 5βP, and high 5βA/5βP and 5αA/5βP ratios.**

© 2001 American Association for Clinical Chemistry

A critical issue in doping control is establishing the origin of testosterone and other steroids typically found in human urine. If the origin can be shown to be pharmaceutical, as opposed to endogenous, a doping offense has occurred. Androgens for pharmaceutical use are not synthesized de novo; they are obtained by semisynthesis from starting materials such as diosgenin and stigmasterol, which are derived from plants *(1)*. Furthermore, these compounds have less ^{13}C than their endogenous homologs *(2, 3)*; therefore, urinary steroids with a low $^{13}C/^{12}C$ ratio are likely to have originated from pharmaceutical sources. This thesis has led to the use of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)[3] in doping control because the method determines the $\delta^{13}C$ value of steroids extracted from urine, based on the equation:

[1] UCLA Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology and [2] Department of Medicine, University of California at Los Angeles, Los Angeles, CA 90025.

*Address correspondence to this author at: UCLA Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology, University of California at Los Angeles, 2122 Granville Ave., Los Angeles, CA 90025-6106. Fax 310-206-9077; e-mail rodrigoa@ucla.edu.

Received June 7, 2000; accepted November 22, 2000.

[3] Nonstandard abbreviations: GC-C-IRMS, gas chromatography-combustion-isotope ratio mass spectrometry; T/E, testosterone/epitestosterone ratio; 5βA, $\delta^{13}C$ value for 5β-androstane-3α,17β-diyl diacetate; 5αA, $\delta^{13}C$ value for 5α-androstane-3α,17β-diyl diacetate; 5βP, $\delta^{13}C$ value for 5β-pregnane-3α,20α-diyl diacetate; and QC, quality control.

USADA 1225

$$\delta^{13}C[‰] = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 1000$$

where $^{13}C/^{12}C$ refers to the isotope ratio in the sample or an international standard (a reference gas calibrated relative to Pee Dee Belemnite). Accordingly, for several years, GC-C-IRMS has been applied to detect the use of exogenous steroids such as testosterone *(3, 4)*, dihydrotestosterone *(5, 6)*, and dehydroepiandrosterone *(6, 7)* by athletes.

Testosterone administration increases the ratio of urine testosterone to epitestosterone (T/E). This finding led to the use of T/E as a screening test for testosterone administration *(8)*. If the ratio exceeds 6, laboratories accredited by the International Olympic Committee report the result to the sport authority *(9)*. It is known, however, that factors other than testosterone administration may also increase the T/E ratios *(10–12)*. Furthermore, testosterone administration does not always lead to an elevated[4] T/E ratio *(3, 13)*. These factors complicate the interpretation of elevated T/E ratios and limit the significance of T/E ratios <6. Determining the T/E time profile of an individual is useful, but it requires consideration of the results of past tests or the collection of additional samples. Our previous work demonstrated that the measurement of $\delta^{13}C$ values of testosterone and its metabolites can detect testosterone use *(4, 14, 15)*. Others have reported that $\delta^{13}C$ values may be abnormally low even in samples with T/E ratios <6 *(3)*.

With the introduction of new methods, it is essential to fully characterize the assay and to establish the values in the control group. This is particularly true with $\delta^{13}C$ measurements when applied to drug control because the method is inherently difficult, the analytical results are likely to be litigated, and it has been suggested *(3, 5)* that diet or ethnicity may influence the $^{13}C/^{12}C$ ratio of urine steroids. Accordingly, herein we characterize the $\delta^{13}C$ measurements of the diacetates of 5β- and 5α-androstane-3α,17β-diol (5βA and 5αA, respectively), and 5β-pregnane-3α,20α-diol (5βP) with respect to precision, linearity, system suitability, and batch acceptance. In addition, we determined $\delta^{13}C$ values for these steroids in a control group of 73 healthy male subjects from four different ethnic groups. Finally, data gathered from the control group were used to interpret $\delta^{13}C$ values obtained on athletes with elevated T/E ratios and to discuss diagnostic criteria for doping.

Materials and Methods

STEROID CALIBRATION SOLUTION

A steroid calibration solution was prepared that contained 25 mg/L 5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol, and 5β-pregnane-3α,20α-diol, all as diacetates (Steraloids, Inc.).

[4] The word "elevated" is used to refer to a result that is above the administrative cutoff.

CONTROL GROUP

The control group consisted of 74 male medical students (age range, 19–29 years) from the University of California at Los Angeles. Body weights were 45–110 kg. The students had the option to give a medical and drug history and to describe their ethnicity. No student declared taking steroids or reported any chronic disease. The ethnicities were "Asian" (n = 16), "African American" (n = 9), "Caucasian" (n = 25), "Hispanic" (n = 13), "other" (n = 7), and "unknown" (n = 4). The students collected 24-h urine samples, and 50-mL aliquots were stored at 4 °C. The study was conducted under the guidance of the Human Subject Protection Committee of the University of California at Los Angeles.

ATHLETES WITH ELEVATED T/E RATIOS

Of the large number of anonymous urine samples that our laboratory analyzes for sport drug control programs in the US, some have a urine T/E ratio >6. In such cases, the sport organization typically collects additional samples to track the urine T/E over weeks or months. We selected some of these urine samples for GC-C-IRMS analysis based on the availability of at least two samples per person, sufficient volume to perform the analysis, and permission from the sport organization.

QUALITY-CONTROL URINE SAMPLES

Quality-control urines (QC-H and QC-L) were obtained from two subjects known not to be using testosterone or any substance likely to alter the $\delta^{13}C$ values of 5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol, and 5β-pregnane-3α,20α-diol (measured as diacetates). Each subject donated one 8-h urine, which was aliquoted into 10-mL cryogenic tubes and stored at −20 °C until analysis.

URINARY STEROID CONCENTRATIONS AND SAMPLE PREPARATION FOR GC-C-IRMS

The T/E ratio was estimated by GC-MS as described previously *(16)*. The concentrations of 5β- and 5α-androstane-3α,17β-diol were estimated from a QC sample prepared by adding to steroid-free urine 5β- and 5α-androstane-3α,17β-diol. [16,16,17-^{2}H]-testosterone (CDN Isotopes) was used as an internal standard. The 5β-pregnane-3α,20α-diol was not quantified. The sample preparation methods for GC-C-IRMS analysis and the instrument conditions have been described *(15)*. The volume of urine extracted for GC-C-IRMS was 10 mL.

GC-C-IRMS DETERMINATION OF 5αA, 5βA, AND 5βP

The GC-C-IRMS analyses were conducted on a Finnigan Delta Plus Isotope Ratio Mass Spectrometer. The IRMS was connected to a Hewlett Packard Model 6890 gas chromatograph via a Finnigan Combustion III interface. The GC was equipped with an HP-50+ capillary column [30 m × 0.25 mm (i.d.); 0.15 μm film thickness] and a Finnigan A200S autosampler. The combustion oven was

oxidized by back-flushing with oxygen for 1 h every 30 samples. The oxidation reactor in the combustion oven was replaced every 500 samples. The injection volume was 1 μL. The recoveries for 5β- and 5α-androstane-3α,17β-diol were 85% and 86%, respectively.

GC-C-IRMS RESPONSE LINEARITY

The linearity of the GC-C-IRMS response was determined by preparing seven solutions containing all three analytes at 2.5, 5, 10, 25, 50, 100, and 150 mg/L, respectively. One microliter of each solution was injected three times on 1 day, and the three $\delta^{13}C$ values for each compound were averaged.

PRECISION STUDIES

Instrument precision for 5βP, 5βA, and 5αA was determined by extracting one aliquot of each QC urine and injecting each extract four times in succession. The within-day precision was determined by extracting 20 aliquots of QC-H in the same batch and injecting each one once. The between-assay precision was determined by extracting one aliquot of QC-H and QC-L per day for 16 days, spanning 15 months, and injecting each aliquot once.

DAILY SYSTEM SUITABILITY TEST

To establish a tolerance range, the steroid calibrator was injected five times on each of 5 different days over 2 weeks (n = 25), and the mean $\delta^{13}C$ values and SDs were calculated for each steroid. Each day, before urine sample analysis, the system suitability was assessed by injecting the calibrator three times and calculating the mean $\delta^{13}C$ values. The system was considered suitable for batch analyses if the mean $\delta^{13}C$ values of at least two of the three steroids were within ± 2 SD of the means described above. If the system suitability test failed, maintenance was performed on the gas chromatograph injection port, the GC column and/or the oxidation reactor were replaced, and the calibrator was re-injected. Data acceptance criteria included absence of peak tailing, retention times of the steroids within ± 1% of established values, and a minimum 0.8 V response at *m/z* 44 for each steroid.

CRITERIA FOR BATCH ACCEPTANCE

To establish a tolerance range for the QC urines for batch data acceptance, two aliquots of each QC urine were extracted and each injected twice per day for 5 days spanning 2 weeks (n = 20). ANOVA was performed using the factors day (5), injection (2), duplicate (2), and QC (2). The mean $\delta^{13}C$ values and SDs were calculated for each of the three steroids. For batch analysis, each unknown urine sample was extracted and injected once. One aliquot of the two QC urines and one aliquot of the steroid calibrator were analyzed with each batch of samples. These three controls were injected at the beginning, in the middle, and at the end of the batch. The batch was accepted if at least six of the nine means for the three steroids were within ± 2 SD of the previously established means. The acceptance criteria were as noted above except that an instrument response of 0.3 V at *m/z* 44 was accepted.

STATISTICAL ANALYSES

The Smirnov-Grubbs method was used to test the control group for outliers; otherwise, all statistical tests utilized statistical software (SAS). The linearity of the GC-C-IRMS response was assessed by least-squares linear regression. The response was considered linear if the slope was zero at $P = 0.01$. The method of Koch and Peters *(17)* was used to determine the SDs of duplicates. The normality of the distributions of 5αA, 5βA, and 5βP, differences, and ratios was determined by the Anderson-Darling test. The values for 5αA, 5βA, and 5βP were correlated with Pearson's correlation coefficient, *r*. Two-sided paired *t*-tests were used to compare the means of 5αA, 5βA, and 5βP in the control group. The general linear model procedure was used to assess differences between the mean $\delta^{13}C$ values of the ethnic groups, and the power of the analysis was assessed by one-way ANOVA.

Results

LINEARITY OF GC-C-IRMS RESPONSE

The IRMS response was linear for 5β- and 5α-androstane-3α,17β-diyl diacetate from 2.5 to 150 mg/L (Fig. 1). However, for 5β-pregnane-3α,20α-diyl diacetate, the response was linear only between 5 and 150 mg/L. The concentrations of 5β- and 5α-androstane-3α,17β-diol in the control group were 23–430 and 25–204 μg/L, respectively. Therefore, assuming 85% recovery, the amounts of 5β- and 5α-androstane-3α,17β-diol extracted from the 10-mL urine and reconstituted in 25 μL of cyclohexane were analyzed in the linear range of the IRMS.

PRECISION OF THE GC-C-IRMS INSTRUMENT AND ASSAY

Instrument precision (SD) for 5βA in QC-H and QC-L was 0.32‰ and 0.41‰, respectively. For 5αA, it was 0.08‰

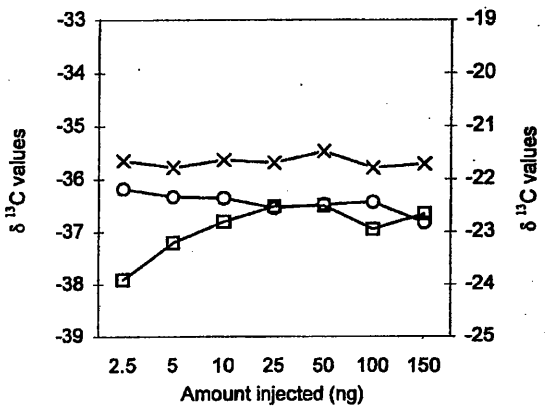


Fig. 1. Relationships between concentration of 5β-androstane-3α,17β-diyl diacetate (×), 5α-androstane-3α,17β-diyl diacetate (○), 5β-pregnane-3α,20α-diyl diacetate (□; *right axis*) and $\delta^{13}C$ values.

and 0.59‰, respectively, and for 5βP, it was 0.27‰ and 0.16‰, respectively. The descriptive statistics for the within-assay experiment on QC-H are shown in Table 1. The SDs were 0.27‰, 0.38‰, and 0.28‰ for 5βA, 5αA, and 5βP, respectively, and the CVs were ≤1.4%. The range of values was 0.9‰ for 5βA, 1.2‰ for 5βP, and 1.8‰ for 5αA. The between-assay SDs for QC-H were 0.40‰, 0.42‰, and 0.44‰ for 5βA, 5αA, and 5βP, respectively, and the CVs were ≤1.8% (Table 2). For QC-L, the values were slightly higher. The mean $\delta^{13}C$ values for the three steroids in QC-L were significantly lower than the means for QC-H.

SYSTEM SUITABILITY AND BATCH ACCEPTANCE

In the system suitability test, the SDs of 5βA, 5αA, and 5βP were 0.31‰, 0.59‰, and 0.54‰, respectively. The tolerance range for the batch acceptance data provided SDs for 5βA, 5αA, and 5βP of 0.68‰, 0.67‰, and 0.65‰ for QC-H, and 0.66‰, 0.56‰, and 0.39‰ for QC-L, respectively. Because these data consisted of duplicate analyses, we calculated the SDs of duplicates and performed an ANOVA. The SDs of duplicates were 0.24‰, 0.32‰, and 0.37‰ for 5βA, 5αA, and 5βP, respectively. These values were approximately one-half the SDs obtained for the factor injection. ANOVA revealed that QC-H and QC-L were different, and there was no difference for the factors injection, duplicate, or day.

CONTROL SUBJECTS: DESCRIPTIVE STATISTICS FOR THE CONCENTRATIONS AND $\delta^{13}C$ OF URINARY STEROIDS

The geometric means for urine testosterone, epitestosterone, and 5β- and 5α-androstane-3α,17β-diol concentrations were 20, 29, 98, and 58 μg/L, respectively. The geometric mean for urine T/E was 0.7 (arithmetic range, 0.2–5.3). The concentration of urine 5β-pregnane-3α,20α-diol was not determined. Table 3 and Fig. 2 show the descriptive statistics and the histograms for 5βA, 5αA, and 5βP. The outlier test was applied, and no data points were excluded. The distributions of 5αA and 5βP were gaussian ($A^2 = 0.28$, $P > 0.25$; and $A^2 = 0.21$, $P > 0.25$, respectively). The distribution of 5βA was gaussian at $P = 0.01$, but not gaussian at $P = 0.05$, $A^2 = 0.85$.

There were significant ($P < 0.0001$) correlations between 5βA and 5αA ($r = 0.70$), between 5βA and 5βP ($r = 0.67$), and between 5βP and 5αA ($r = 0.58$). The CVs of the $\delta^{13}C$ values for the diacetates were 2.6–3.6%. The means − 3 SD for 5βA, 5αA, and 5βP were −28.46‰, −28.39‰, and −26.37‰, respectively. The mean 5αA was lower (−26.35‰) than the mean 5βA (−25.69‰; $P < 0.001$). The Wilcoxon nonparametric two-sample test confirmed that 5αA was lower than 5βA. The means for 5βA and 5αA were lower than the mean for 5βP (both $P < 0.001$).

Table 3 also summarizes the statistics of the differences between the means of 5βP and 5βA (5βP − 5βA) and 5βP and 5αA (5βP − 5αA), and the ratios 5βA/5βP and 5αA/5βP. The distributions of 5βP − 5βA and 5βP − 5αA were gaussian ($A^2 = 0.23$ and 0.19, respectively). The 5βP − 5βA differences ranged from −0.08‰ to 3.17‰, and the mean + 3 SD was 3.47‰. The 5βP − 5αA differences ranged from 0.16‰ to 3.72‰, and the mean + 3 SD was 3.99‰. The distributions of 5βA/5βP and 5αA/5βP were gaussian ($A^2 = 0.19$ and 0.21, respectively). The means of 5βA/5βP and 5αA/5βP were 1.06 and 1.09, respectively, and their corresponding means + 3 SD were 1.14 and 1.17. There were no differences between ethnic groups for the means of 5αA, 5βA, or 5βP (Table 4).

Table 1. Within-assay precision for 5βA, 5αA, and 5βP.

	QC-H		
	5βA	5αA	5βP
Mean, ‰ (n = 20)	−24.46	−26.83	−23.13
SD, ‰	0.27	0.38	0.28
CV, %	1.1	1.4	1.2
Minimum, ‰	−24.90	−27.81	−23.62
Maximum, ‰	−23.96	−26.05	−22.47
Range, ‰	0.9	1.8	1.2

$\delta^{13}C$ VALUES IN ATHLETES WITH ELEVATED T/E RATIOS

The urine concentrations of testosterone and epitestosterone, the T/E ratios, and the $\delta^{13}C$ values for the six male athletes with urine T/E ratios >6 are summarized in Table 5. Of the six, athletes 1 and 2 were known to be taking testosterone for medical reasons. According to the sport organizations ordering the tests, athletes 3–6 denied taking any substance known to influence the T/E ratio. The number of samples per athlete ranged from three to eight. The T/E ratio of all samples was >6 for four of the six athletes. Athlete 4 had one T/E ratio of 5.0, and athlete 5 had one T/E ratio of 5.1; otherwise, all T/E ratios were >6.

Table 5 reveals that for athletes 1–3, all 5βA and 5αA values were lower than the mean − 3 SD values of the controls in Table 3, whereas the 5βP values were all close to the mean 5βP value of the controls (−24.26‰). The cells in Table 5 that are outside the ± 3 SD values of Table 4 are outlined or shaded to facilitate comparisons. The combination of 5βP values that are near the mean and low 5βA and 5αA values produce large differences in 5βP −

Table 2. Between-assay precision for 5βA, 5αA, and 5βP.

	QC-H			QC-L		
	5βA	5αA	5βP	5βA	5αA	5βP
Mean, ‰	−24.72	−26.11	−24.00	−26.99	−27.57	−25.97
SD, ‰	0.40	0.42	0.44	0.50	0.47	0.52
CV, %	1.6	1.6	1.8	1.8	1.7	2.0
Minimum, ‰	−25.43	−27.19	−24.47	−27.65	−28.20	−26.86
Maximum, ‰	−24.06	−25.44	−22.89	−25.69	−26.55	−24.85
Range, ‰	1.4	1.8	1.6	2.0	1.7	2.0
n	15	14	16	15	14	16

USADA 1228

Table 3. Control group of 73 subjects: Descriptive statistics for $\delta^{13}C$, differences between the means, and ratios of the means.

	$\delta^{13}C$, ‰			Difference, ‰		Ratio	
	5βA	5αA[a]	5βP[b]	5βP − 5βA	5βP − 5αA	5βA/5βP	5αA/5βP
Mean	−25.69	−26.35	−24.26	1.43	2.09	1.06	1.09
SD	0.92	0.68	0.70	0.68	0.63	0.028	0.027
CV, %	3.6	2.6	2.9			2.7	2.5
Mean + 3 SD	−22.92	−24.31	−22.15	3.47	3.99	1.14	1.17
Mean − 3 SD	−28.46	−28.39	−26.37	−0.62	0.18	0.97	1.00
Maximum	−23.90	−24.55	−22.92	3.17	3.72	1.13	1.16
Minimum	−27.82	−27.89	−25.49	−0.08	0.16	1.00	1.01
Max − Min	3.9	3.3	2.6				

[a] Mean significantly different from 5βA.
[b] Mean significantly different from 5βA and 5αA.

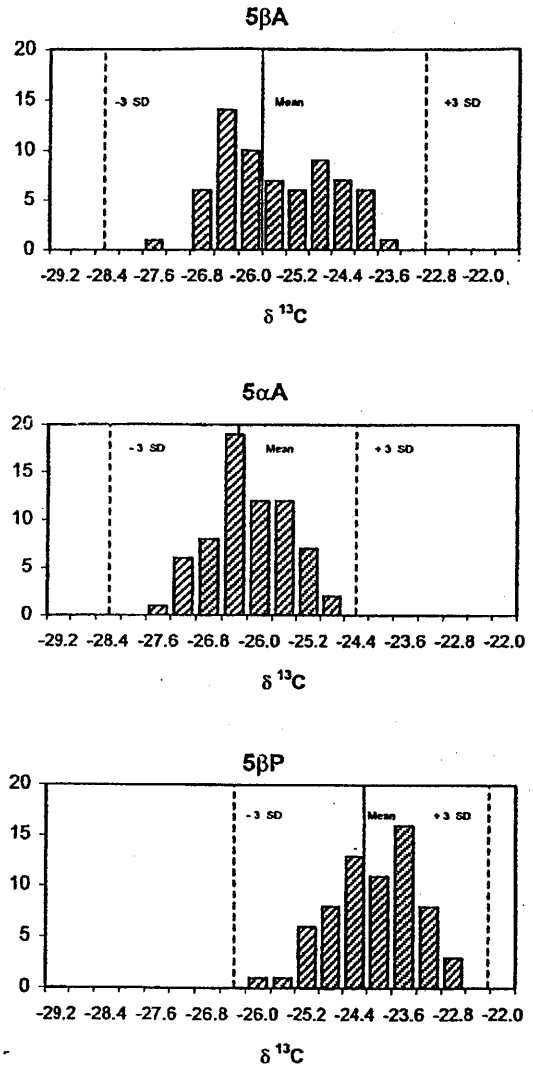


Fig. 2. Histograms of $\delta^{13}C$ values for 5βA (*top*), 5αA (*middle*), and 5βP (*bottom*) for a control group of 73 healthy males.

5βA and 5βP − 5αA, and large 5βA/5βP and 5αA/5βP ratios. For athletes 1–3, all differences and ratios were outside the + 3 SD range. The means of athletes 1–3 and 4–6 and their *z*-scores are presented at the bottom of Table 5. Another aspect of athletes 1–3 was that all 5αA values were lower than the 5βA values for the same urine: the means were −29.84‰ for 5βA and −31.83‰ for 5αA ($P = 0.04$). The means of 5βP − 5βA (5.9‰; *z*-score = 6.5) and of 5βP − 5αA (7.9‰; *z*-score = 9.1) were substantially greater than the + 3 SD values determined for the control group. Similarly, the mean 5βA/5βP ratio was 1.25 (*z*-score = 6.6), and the mean 5αA/5βP ratio was 1.33 (*z*-score = 8.9).

To compare the testosterone concentrations in Table 5 to the general population of athletes that we routinely test, we determined the log mean (3.22 μg/L) and log SD (1.19) of the distribution of the latest 11 938 male urine samples tested in our laboratory. The urine testosterone concentrations in subjects 1 and 2, the two permitted testosterone users, ranged from a low of 2.3 μg/L on the first urine ever collected to 186 μg/L (*z*-score = 1.5, in log

Table 4. Control group: Descriptive statistics for 5βA, 5αA, and 5βP by ethnicity.

Steroid	Ethnicity	n	Mean, ‰	SD, ‰	CV, %
5βA	Cauc.[a]	25	−25.74	0.95	3.7
	Asian	15	−26.17	0.66	2.5
	Hispanic	13	−25.02	1.03	4.1
	A.A.	9	−25.56	0.82	3.2
5αA	Cauc.	25	−26.43	0.79	3.0
	Asian	15	−26.60	0.49	1.8
	Hispanic	13	−26.06	0.73	2.8
	A.A.	9	−26.25	0.79	3.0
5βP	Cauc.	25	−24.34	0.70	2.9
	Asian	15	−24.19	0.68	2.8
	Hispanic	13	−24.14	0.92	3.8
	A.A.	9	−24.22	0.70	2.9

[a] Cauc., Caucasian; A.A., African American.

Table 5. Concentrations of testosterone and epitestosterone; T/E ratios; $\delta^{13}C$ values for 5βA, 5αA, and 5βP; difference scores between 5βP and testosterone metabolites (5βA and 5αA); and ratios of metabolites to 5βP (5βA/5βP and 5αA/5βP) for multiple urine samples obtained from two athletes permitted to take testosterone for medical purposes and four athletes whose drug-taking behavior was unknown.

					$\delta^{13}C$[c]			Difference,[c] ‰		Ratios[c]	
Athlete	Day[a]	[T],[b] μg/L	[E], μg/L	T/E	5βA	5αA	5βP	5βP − 5βA	5βP − 5αA	5βA/5βP	5αA/5βP
1	0	2.3	~0.1	20[d]	NA[e]	NA[e]	NA[e]				—
	1	40	1.0	40	−30.42	−31.96	−25.67	4.8	6.3	1.19	1.25
	4	186	2.6	72	−31.10	−31.76	−24.57	6.5	7.2	1.27	1.29
2	0	27	0.9	29	−31.43	−34.57	−26.14	5.3	8.4	1.20	1.32
	28	100	5.1	20	−28.56	−31.04	−24.00	4.6	7.0	1.19	1.29
	63	56	5.7	9.8	−28.66	−30.76	−22.97	5.7	7.8	1.25	1.34
3	0	343	4.3	80	−28.76	−31.25	−23.06	5.7	8.2	1.25	1.36
	56	690	8.9	77	−28.64	−31.10	−22.60	6.0	8.5	1.27	1.38
	70	598	13.7	44	−29.54	−32.30	−22.30	7.2	10.0	1.32	1.45
	156	820	14.6	56	−29.84	−31.33	−21.67	8.2	9.7	1.38	1.45
4	0	312	29.9	10.4							
	273	128	0.5	10.9	−25.32	−25.76	−24.54	0.8	1.2	1.03	1.05
	357	197	20.4	9.7	−24.96	−26.07	−23.43	1.5	2.6	1.07	1.11
	393	166	24.6	6.8							
	428	394	41.3	9.5	−23.55	−26.14	−22.47	1.1	3.7	1.05	1.16
	461	81	16.0	5.0							
	592	383	43.0	8.9							
	656	211	24.8	8.5							
5	0	108	8.6	12.6							
	64	33	3.5	9.4	−24.82	−25.47	−23.49	1.3	2.0	1.06	1.08
	345	130	15.0	8.6	−24.69	−25.52	−23.27	1.4	2.3	1.06	1.10
	412	36	3.7	9.8	−26.91	−24.50	−24.85	2.1	−0.4	1.08	0.99
	436	35	3.8	9.1							
	798	59	11.4	5.1	−26.66	−27.29	−24.77	1.9	2.5	1.08	1.10
6	0	49	6.3	7.8							
	38	46	6.5	7.1	−24.62	−26.04	−23.36	1.3	2.7	1.05	1.11
	73	11	1.3	8.4							
	103	92	10.3	9.0	−25.12	−25.04	−23.52	1.6	1.5	1.07	1.06
Means, athletes 1–3					−29.84	−31.83	−23.97	5.9	7.9	1.25	1.33
Means, athletes 4–6					−25.08	−25.74	−23.67	1.4	2.1	1.06	1.09
z-Scores, athletes 1–3					4.5	8.0		6.5	9.1	6.6	8.9
z-Scores, athletes 4–6					0.7	0.9		−0.02	−0.02	0.30	0.40

[a] Day of urine collection relative to the first sample (day = 0).
[b] [T] and [E], testosterone and epitestosterone.
[c] Cells >3 SD, shaded; cells <3 SD, solid outline.
[d] Value is an approximation because of large error in measurement of epitestosterone.
[e] Unable to measure because of low response (<0.3 V).

units). For subject 3, the urine testosterone concentrations (mean, 613 μg/L; *z*-score = 2.6) and T/E ratios (mean = 64) were very high. In addition, like athletes 1 and 2, athlete 3 was characterized by low 5βA and 5αA values, and large differences and ratios outside the ± 3 SD range.

In contrast to athletes 1–3, for athletes 4–6, the values for 5βA, 5αA, and 5βP and their differences and ratios were similar to the averages for the control group, and none of the values were remarkable except for one 5βP − 5αA value of −0.4‰ and one 5αA/5βP ratio of 0.99, which were slightly lower than the mean − 3 SD. For subjects 4–6, 5βP − 5βA and 5βP − 5αA were less than 2.1‰ and 3.7‰, respectively, i.e., within ± 3 SD of the control group values. Similarly, all but one of the ratios to 5βP were inside the ± 3 SD range. In addition, the urine testosterone concentrations for subjects 5 and 6 were within + 1.5 SD of the log mean for 11 938 athletes. Thus, compared with the control group, athletes 5 and 6 were characterized by elevated T/E ratios; whereas the values for 5βA, 5αA, and 5βP, their differences, and their ratios for athletes 5 and 6 were, with two minor exceptions, within ± 3 SD of the means of the controls. Athlete 4 had a relatively high mean urine testosterone (234 μg/L; *z*-score = 1.9).

The use of testosterone or testosterone precursors is indicated by low 5αA and 5βA values, large 5βP − 5αA

and 5βP − 5βA differences, and large 5αA/5βP and 5βA/5βP ratios. Of these six variables (two $\delta^{13}C$ values, two differences, and two ratios), for athletes 1–3, the ratio 5βA/5βP (z-score = 8.9) and the difference 5βP − 5βA (z-score = 9.1) were the most sensitive indicators of testosterone administration as judged by z-scores. The value for 5αA was also a good indicator (z-score = 8.0). For differences and ratios that utilized 5βA (5βP − 5βA and 5βA/5βP), the z-scores were 6.5 and 4.5, respectively. The least discriminating variable was 5βA (z-score = 4.5). In contrast, for athletes 4–6 in Table 5, the z-scores were all low (range, −0.02 to 0.9).

Discussion

CHARACTERISTICS OF THE GC-C-IRMS ASSAY

GC-C-IRMS measurement of urinary steroids has become a potent analytical method in doping control, although scrupulous attention to detail is necessary. Successful implementation of the method requires strict adherence to QC, system suitability, and batch acceptance criteria such as those described herein. In the present study, after the tolerance ranges for the system and batches were established, the system suitability test never failed and the batch acceptance criteria were always met over the 14 months of the study.

In the instrument precision study, the mean SDs were less than ± 0.33‰; thus, when the variability attributable to extraction and derivatization was eliminated, all of the SDs were low. In the within-assay study, the SDs were also low: ± 0.27‰, ± 0.38‰, and ± 0.28‰ for 5βA, 5αA, and 5βP, respectively. The between-assay SDs ranged from ± 0.40‰ to ± 0.52‰, which is slightly higher than the within-assay values, but they indicate the excellent repeatability and reproducibility of the entire assay.

We did not find comparable precision studies for urinary steroids in the literature; however, Shackleton et al. *(3)* provided evidence that $\delta^{13}C$ values for diols are reproducible based on duplicate analysis of 13 samples from one subject. We used the data in Table 1 from Shackleton et al. *(5)* to calculate the SD of duplicates and found values of ± 0.36‰, ± 0.15‰, and ± 0.28‰ for 5βA, 5αA, and 5βP, respectively. These data show that the SD for duplicates in another laboratory was similar to the SD that we obtained for duplicates in our batch acceptance studies. The concentrations of 5β-androstane-3α,17β-diol and 5α-androstane-3α,17β-diol in the control urines were 23–430 μg/L; thus, given the 85% recovery of steroids from the 10-mL sample volume used in the analysis, the concentrations of 5β-androstane-3α,17β-diol and 5α-androstane-3α,17β-diol in the 25 μL of the injection solution were 8–146 mg/L, and the samples were analyzed in the linear range of the system (Fig. 1).

HEALTHY CONTROL MALE SUBJECTS

The $\delta^{13}C$ values in Table 3 represent the largest group of healthy males living in the US measured to date. The ranges for the T/E ratios (0.1–3.6) and urine testosterone concentrations (2–116 μg/L) are consistent with the fact that no student declared taking testosterone or a steroid supplement. In addition, none of the students declared any endocrine disorders. Of the 74 urines, we were able to measure 5βA, 5αA, and 5βP values for all but 1. Sixty-eight of the samples provided satisfactory data on the first extraction of 10 mL of urine. For the other six, three provided an adequate signal on repeat analysis. Two samples required a 20-mL sample volume to obtain an adequate signal. In one sample with low diol concentrations (<25 μg/L), the voltage criteria were not met even with a 20-mL sample. Thus, our signal criteria were met on the first analysis with 10 mL of urine in 94% of the 74 cases, and all but 1 of the remaining samples provided satisfactory data on repeat analysis with 10 or 20 mL of urine. One person with one instrument can perform approximately three assays per week with a batch size of 20 samples plus 5 controls. The software to process the samples is somewhat cumbersome; thus, ~2 days per week are needed for data processing.

The mean 5αA and 5βA values for our control group were −26.35‰ and −25.69‰, respectively. The mean δ values − 3 SD for 5βA and 5αA were approximately −28.4‰, which is similar to the δ values reported for synthetic testosterone *(6, 7)*; however, this comparison should not be made. This is because the diols measured herein were acetylated, which makes the δ value more negative by approximately two units, whereas the synthetic testosterone *(6, 7)* was underivatized.

There are no comparable control group values in the literature; however, the baseline values in Fig. 6 of Shackleton et al. *(3)*, which was based on analysis of 20 samples from individuals with mixed nationalities, are very similar to ours. Their lowest value for a diol was −28.2‰, whereas our lowest diol value was −27.89‰. Ueki and Okano *(7)* also measured 5αA and 5βA; however, their data are not directly comparable to ours because they used a correction factor to convert all measured $\delta^{13}C$ values of the acetylated compounds to give values for underivatized steroids, which we could not estimate. The correction factor would be the same for 5αA and 5βA; thus, their difference of 2.6‰ between the means of 5αA (−16.4‰) and 5βA (−19.0‰) for 10 Japanese male samples can be compared to our difference of 0.66‰ (Table 3). In addition, the value for 5αA was lower than 5βA in the study by Ueki and Okano *(7)*, which is the opposite of what we found. We can also compare the range of values in our control subjects to the range reported by Ueki and Okano *(7)* in their Table 2 for 20 healthy Japanese males. Our ranges for 5αA and 5βA were 2.9‰ and 3.9 ‰ (Table 4), whereas they found ranges of 10.8‰ and 7.2‰, respectively. The striking difference between the ranges found in the two laboratories could reflect differences in method, calibration, ethnicity, or diet. The explanation that Japanese subjects have different steroid biochemistry and metabolism was deemed unlikely because equally large ranges of 11.0‰ (5βA) and 14.1‰ (5αA) were

reported for urine samples from >350 Olympic athletes, and Olympic athletes are multiracial. In addition, Shackleton et al. *(3)* found similar values for Chinese and other nationalities, and we found no difference among four ethnic groups in our control group. That dietary factors could explain the differences was also considered, but diet is an unlikely explanation for the large differences in Olympic athletes because it is likely to take several weeks on a local diet before a measurable change in urinary steroid $\delta^{13}C$ develops. Therefore, it is likely that our analytical method differs from that of Ueki and Okano *(7)*.

The diversity of the control group was an advantage in that four major ethnic groups were present; however, it was a disadvantage because the n for each ethnic group was relatively small and therefore the statistical power of the GLM procedure was relatively low. Thus, we could not attribute any differences to ethnicity. Similarly, Shackleton et al. *(5)*, who compared $\delta^{13}C$ values for 20 individuals from 12 nationalities, did not attribute any of the differences to ethnicity or diet.

In the present study, the mean $\delta^{13}C$ values for 5αA (−26.35‰) and 5βA (−25.69‰) in the control group were different (P <0.001), whereas in our previous study with only 10 subjects, we did not observe such a difference *(15)*. In addition, it appears from the data in Table 5 of the present study and from Table 1 in our previous study *(15)* that testosterone administration may decrease 5αA more than it decreases 5βA. This difference occurs despite the fact that the percentage of conversion of a tracer dose of [^{14}C]testosterone to 5β-androstane-3α,17β-diol (3.2%) is greater than the percentage of conversion to 5α-androstane-3α,17β-diol (1.2%) *(18)*. Urinary 5α-androstane-3α,17β-diol is known to arise from both hepatic and peripheral metabolism, whereas urinary 5β-androstane-3α,17β-diol is considered to arise only from hepatic metabolism *(19)*. After pharmaceutical doses of testosterone, 5αA may decrease more than 5βA because peripheral paths to 5α-androstane-3α,17β-diol are favored. However, there is also a path from dehydroepiandrosterone sulfate to 5β-androstane-3α,17β-diol that does not pass through testosterone *(20)*. Utilization of this path could explain why 5βA is higher than 5αA. Finally, the observed differences may be an analytical artifact. Similarly, the higher mean $\delta^{13}C$ value for 5βP (−24.26‰) might be related to metabolism or artifacts.

ATHLETES WITH ELEVATED T/E RATIOS

To clarify the status of an athlete with an elevated T/E ratio, the International Olympic Committee recommends that additional samples be obtained *(9)*. The relevant sport authority typically charts the course of the urine T/E values over time and interprets the data pattern to decide whether to penalize the athlete. Unless there is a record of three or more past samples, this involves collecting additional samples. There are no guidelines stating how many samples are needed, how often they should be collected, and for how long, and there are no published criteria for determining whether the elevated T/E ratio is natural or is attributable to taking an exogenous substance. The data in Tables 1–3 and 5 support the argument that by measuring $\delta^{13}C$, the decision to penalize the athlete can be made on a single sample. The six athletes described in Table 5 were undergoing such testing, although at the time of the analysis the laboratory was not aware that subjects 1 and 2 were permitted testosterone users. No additional information was available on the status of athletes 3–6; however, inspection of the data for subject 3 indicates that it is reasonable to classify him as a testosterone or testosterone precursor user.

The six subjects in Table 5 with increased T/E ratios may be characterized by the concentration of testosterone in their urines and by their 5βA and 5αA values and the associated differences and ratios to 5βP. A classification based on $\delta^{13}C$ values, differences, and ratios revealed that athletes 1–3 were outside the control range ± 3 SD except for 5βP. For athletes 1 and 2, this fits with our understanding of the basis of the carbon isotope ratio method, specifically, that after testosterone administration only the $\delta^{13}C$ values of testosterone and testosterone metabolites will decrease and that those of other urinary steroids will not. Because 5β-pregnane-3α,20α-diol is not a metabolite of testosterone, its $\delta^{13}C$ values are not expected to change with testosterone administration *(3, 4, 15)*.

Applying the means ± 3 SD criteria to the $\delta^{13}C$ data for athlete 3 requires classifying him as a testosterone or testosterone precursor user. This possibility is further supported by the very high urine T/E ratios and testosterone concentrations. The *z*-scores for his urine testosterone concentrations range between 2.2 and 2.9, which is a further indication of testosterone or testosterone precursor administration. Once the GC-C-IRMS method is validated to the point of full acceptance by the sport and legal community, we expect that athlete 3 could be classified as a user on one urine showing the pattern of a high T/E ratio, high testosterone concentration, and $\delta^{13}C$ values less than − 3 SD.

Athletes with naturally elevated urine T/E ratios would be expected to have persistently elevated T/E ratios, unremarkable urine testosterone concentrations, and values for 5βA and 5αA that are within ± 3 SD of the mean of the control group. The data for athletes 5 and 6 fit this pattern. Athlete 4 also fits, but some of his urine testosterone concentrations were relatively high. The *z*-score for the values 383 and 394 μg/L was ~2.3, and all but one of the others were >1.3. Until additional data are available, athlete 4 has been classified as having a naturally elevated T/E ratio, although we cannot completely exclude the possibility that athlete 4 is a testosterone or testosterone precursor user with normal $\delta^{13}C$ values.

The data on mean *z*-scores in Table 5 reveal that both the differences and ratios are better indices of testosterone use than the absolute $\delta^{13}C$ values, 5βA and 5αA. This is

not unexpected given that 5βP correlates with both 5βA and 5αA. Furthermore, the difference 5βP − 5βA and the ratio 5βA/5βP are more robust indicators of testosterone use than the corresponding differences 5βP − 5αA and the ratio 5αA/5βP. This follows from the finding (Tables 3 and 5) that the $\delta^{13}C$ values for 5αA were lower than the values for 5βA.

In conclusion, a fundamental issue in doping control is whether GC-C-IRMS techniques will resolve ambiguities in the interpretation of the T/E test. The methods presented herein provide compelling evidence that the test has excellent precision and that when it is coupled with strict system suitability and batch acceptance criteria, it can be used in a routine fashion to obtain valid $\delta^{13}C$ data. By comparing the $\delta^{13}C$ values of a control group to data obtained from athletes, it is possible to make informed decisions regarding the origin of urinary 5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol, and 5β-pregnane-3α,20α-diol.

We are grateful for financial support from the National Collegiate Athletic Association, the National Football League, and the United States Olympic Committee. We thank K. Schramm for technical assistance.

References

1. Coppen JJW. Steroids: from plants to pills—the changing picture. Trop Sci 1979;21:125–41.
2. Smith BN, Epstein S. Two categories of $^{13}C/^{12}C$ ratios for higher plants. Plant Physiol 1971;47:380–4.
3. Shackleton CHL, Phillips A, Chang T, Li Y. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids 1997;62:379–87.
4. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG. Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. J Mass Spectrom 1996;31:169–76.
5. Shackleton CH, Roitman E, Phillips A, Chang T. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: potential use in gas chromatography isotope ratio mass spectrometry. Steroids 1997;62:665–73.
6. Horning S, Geyer H, Flenker U, Schänzer W. Detection of exogenous steroids by $^{13}C/^{12}C$ analysis. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U, eds. Recent advances in doping analysis, Vol. 5. Köln: Sport und Buch Strauss, 1997:135–48.
7. Ueki M, Okano M. Analysis of exogenous dehydroepiandrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 1999; 13:2237–43.
8. Donike M, Bärwald KR, Klostermann K, Schänzer W, Zimmermann J. Nachweis von exogenem Testosteron. In: Heck H, Hollmann W, Liesen H, Rost R, eds. Sport: Leistung und Gesundheit. (Kongressbd. Deutsche. Sportarztekongress) Köln: Deutsche Ärzte-Verlag, 1983:293–8.
9. International Olympic Committee. List of doping classes and methods. Lausanne, Switzerland: IOC, 1992.
10. Falk O, Palonek E, Björkhem I. Effect of ethanol on the ratio between testosterone and epitestosterone in urine. Clin Chem 1988;34:1462–4.
11. Oftebro H. Evaluating an abnormal urinary steroid profile. Lancet 1992;339:941–2.
12. Catlin DH, Hatton CK, Starcevic S. Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athletes' urine. Clin Chem 1997;43:1280–8.
13. Dehennin L, Matsumoto AM. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. J Steroid Biochem Mol Biol 1993;44: 179–89.
14. Becchi M, Aguilera R, Farizon Y, Flament M-M, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. Rapid Commun Mass Spectrom 1994;8: 304–8.
15. Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, Swerdloff RS, et al. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. J Chromatogr B 1999;727:95–105.
16. Catlin DH, Kammerer RC, Hatton CK, Sekera MH, Merdink JM. Analytical chemistry at the Games of the XXIIIrd Olympiad in Los Angeles, 1984. Clin Chem 1987;33:319–27.
17. Koch DD, Peters TP. Selection and validation of statistical methods. In: Burtis CA, Ashwood ER, eds. Fundamentals of clinical chemistry, 2nd ed. Philadelphia: WB Saunders, 1996:170–81.
18. Baulieu EE, Robel P. Catabolism of testosterone and androstenedione. In: Eik-Nes KB, ed. The androgens of the testis. New York: Marcel Dekker, 1970:49–71.
19. Mauvais-Jarvis P, Floch H, Jung I, Robel P, Baulieu EE. Studies on testosterone metabolism. VI. Precursors of urinary androstanediols. Steroids 1968;11:207–24.
20. Mauvais-Jarvis P, Baulieu EE. Studies on testosterone metabolism. IV. Urinary 5α- and 5β-androstanediols and testosterone glucuronide from testosterone and dehydroisoandrosterone sulfate in normal people and hirsute women. J Clin Endocrinol Metab 1965;25:1167–78.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2007, 55, 197–203 197

Improved Detection of Sugar Addition to Maple Syrup Using Malic Acid as Internal Standard and in ^{13}C Isotope Ratio Mass Spectrometry (IRMS)

PATRICE TREMBLAY† AND RÉAL PAQUIN*,‡

Université du Québec, Institut National de la Recherche Scientifique, Centre Eau, Terre et Environnement, 2800 rue Einstein, C.P. 7500, Sainte-Foy, Quebec, Canada G1P3W8, and Université Laval, Département de Physique, de Génie Physique et d'Optique, Cité Universitaire, Quebec, Canada G1K7P4

Stable carbon isotope ratio mass spectrometry ($\delta^{13}C$ IRMS) was used to detect maple syrup adulteration by exogenous sugar addition (beet and cane sugar). Malic acid present in maple syrup is proposed as an isotopic internal standard to improve actual adulteration detection levels. A lead precipitation method has been modified to isolate quantitatively malic acid from maple syrup using preparative reversed-phase liquid chromatography. The stable carbon isotopic ratio of malic acid isolated from this procedure shows an excellent accuracy and repeatability of 0.01 and 0.1‰ respectively, confirming that the modified lead precipitation method is an isotopic fractionation-free process. A new approach is proposed to detect adulteration based on the correlation existing between the $\delta^{13}C_{malic\ acid}$ and the $\delta^{13}C_{sugars} - \delta^{13}C_{malic\ acid}$ ($r = 0.704$). This technique has been tested on a set of 56 authentic maple syrup samples. Additionally, authentic samples were spiked with exogeneous sugars. The mean theoretical detection level was statistically lowered using this technique in comparison with the usual two-standard deviation approach, especially when maple syrup is adulterated with beet sugar : 24 ± 12% of adulteration detection versus 48 ± 20% (*t*-test, $p = 7.3 \times 10^{-15}$). The method was also applied to published data for pineapple juices and honey with the same improvement.

KEYWORDS: Maple syrup; sugars and malic acid; internal standard; authentification; stable isotope analysis; ^{13}C IRMS; pineapple juices; honey

INTRODUCTION

Maple syrup, which is produced from the xylem sap of *Acer saccharum* March, has recently become a luxury product in many countries. It is thus important to develop an effective and adequate method to detect low levels of adulteration. Maple syrup adulteration can be accomplished by adding inexpensive sugars (mainly sucrose) to maple sap or by simple dilution of maple sap with sugar syrup, in either case for economic gain. Several methods have been developed to detect adulteration (*1*–*5*). Most of them are based on the determination of the stable carbon isotope ratio of the dry maple syrup. This method is applicable because the $^{13}C/^{12}C$ ratio of a plant-derived material reflects the photosynthetic pathways employed by plants during atmospheric CO_2 fixation.

There exist three photosynthetic pathways or cycles: the Calvin cycle (*6*), the Hatch–Slack cycle (*7*) and the crassulacean acid metabolism (*8*). Plants using the Calvin cycle, such as the maple tree and sugar beets, are commonly called C_3-plants. This photosynthetic pathway is recognized to produce organic compounds depleted in ^{13}C (*9*) where the stable carbon isotopic ratio, $\delta^{13}C$, varies between −20 and −32‰ with a mean of −27‰ (relative to the international standard VPDB like all $\delta^{13}C$ presented here). Plants using the Hatch–Slack cycle, like cane sugar, are commonly called C_4-plant, and their $\delta^{13}C$ values range fall between −9 to −17‰ with a mean of −13‰. Finally, plants using the crassulacean acid metabolism are named CAM-plant and have an intermediate stable carbon isotopic composition with values varying between −10 and −20‰.

Adulteration of maple syrup (C_3-plant) by cane sugar (C_4-plant) or beet sugar (C_3-plant) can be detected by carrying out the stable carbon isotopic ratio of the dry maple syrup as shown by the current methods of adulteration detection (*1*–*5*). Their detection limits (varying from 10 to 50% of sugar addition) depend on several factors, particularly on the natural stable carbon isotopic distribution of the authentic product (maple syrup) and of the adulterant (cane or beet sugar). The adulteration detection limits are obtained from the standard deviation of the natural variation of the maple syrup isotopic composition. Lower adulteration detection levels may be achieved using an

* Corresponding author. Tel.: (418) 656-2131 ext. 6237. Fax: (418) 656-2040. E-mail: rpaquin@phy.ulaval.ca.
† Université du Québec.
‡ Université Laval.

10.1021/jf062413a CCC: $37.00 © 2007 American Chemical Society
Published on Web 12/24/2006

USADA 1234

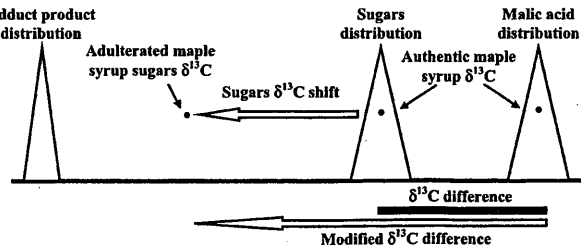


Figure 1. Schematic representation of authentic product adulteration and implication on the $\delta^{13}C$ values of the product and the internal standard and the $\delta^{13}C$ difference between them (product – internal standard).

internal standard, but this technique has not been applied to maple syrup. Therefore, there is a need for new isotopic internal standards to enhance the information provided by $\delta^{13}C$ of the dry maple syrup. Ideally, if the value of the stable carbon isotopic ratio of the original components of the mixture was known, this would be under the best experimental conditions to determine adulteration.

To achieve these conditions, several authors have proposed the use of an internal standard (*10*–, *11*) naturally present in the sample. This internal standard should have the same isotopic characteristics as the original product (information about photosynthetic origin and environmental and physiological behavior). **Figure 1** illustrates the $\delta^{13}C$ shift produced on the product and the isotopic value difference between the product and the internal standard when adulteration occurs. It also demonstrates the potential of internal standard utilization to obtain information about the original $\delta^{13}C$ value of the product if an isotopic correlation relationship exists between the internal standard and the product.

The ideal candidate for such an approach must be easy to isolate in sufficient quantity to be analyzed. Furthermore, it should not be commercially available at an attractive price for use on a large scale (*11*). For food adulteration detection, pulp (*10*, *13*), proteins (*12*, *14*), and malic and citric acid (*17*–*19*) have been used. From the maple syrup constituants (*20*), malic acid seems to be the most interesting internal standard because commercial sweeteners contain very little malic acid (*21*). There are already several methods for malic acid extraction in foods (*22*, *23*). The use of intermolecular stable carbon isotope correlation between sugars and malic acid in fruit juices has been suggested as a criterion for authenticity. The standard deviation of the natural isotopic variation of the internal standard or the isotopic difference between the internal standard and the sugars is used to determine the adulteration detection limits (*17*–*19*). However, maple syrup is produced from the thermal evaporation of the sap water. Complex chemical reactions occur, which may alter the isotopic composition of the sugars and the malic acid and therefore may lower the specificity normally associated with the internal standard procedure used for juice adulteration as juice is produced from a physical process like pressing, which does not produce isotopic fractionation. Moreover, the correlation usually found between the internal standard and the target adulteration specie, mainly sugars, should be used to determine the adulteration limits instead of using the standard deviation on a single isotopic population as used now.

We present herein the results obtained for maple syrup using malic acid as an internal standard. An optimized procedure is described for extraction and separation of malic acid from maple syrup involving a lead precipitation procedure. It will be examined how the isotopic correlation allows a better maple syrup adulteration detection, and a novel calculation approach will be introduced and compared with the current technique for adulteration detection of maple syrup. This proposed approach will also be tested on other foodstuffs using previously published data.

MATERIALS AND METHODS

Apparatus and Reagents. Potassium dihydrogen phosphate (KH_2PO_4) ≥ 99.5% and D-malic acid 99% were purchased from Sigma-Aldrich (Milwaukee, MI). Anhydrous oxalic acid ~97%, shikimic acid >97%, succinic acid >99.5%, citric acid >99.5%, fumaric acid >99.5%, and l(+)-tartaric acid >99.5% were purchased from Fluka (Milwaukee, MI). Sodium hydroxide (NaOH) 98+% A.C.S. was obtained from OMEGA (Levis, Quebec, Canada), lead nitrate ($Pb(NO_3)_2$) A.C.S. from Laboratoire MAT (Montréal, Quebec, Canada), hydrochloric acid (HCl) concentrate A.C.S. from Fisher (Nepean, Ontario, Canada), phosphoric acid (H_3PO_4) 85% from Mallinckrodt (Paris, KY), ethanol 95% from Commercial Alcohols (Montréal, Quebec, Canada), artificial maple flavor 187 M-11C from Richard Facey Ltd. (Rexdale, Ontario, Canada), cane sugar from Lactil (Toronto, Ontario, Canada), and water from a standard Millipore Milli-Q Plus Water system (Bedford, MA) were obtained.

The LC system consisted of a Waters Model 510 LC pump (Waters, Milford, MA), a Model 7725i Rheodyne injector (Supelco, Oakville, Ontario, Canada), a Perkin-Elmer Model LC-55 spectrophotometer (Perkin-Elmer, Nornalk, CT), and a HPChem data acquisition station (Agilent Technology, Montréal, Quebec, Canada). A 250 mm × 21.21 mm i.d., 5 μm Phenomenex Maxcil C_{18} column and a 30 mm × 4.60 mm i.d., 5 μm Phenomenex Maxcil C_{18} guard column were used for preparative chromatography (Phenomenex, Torrance, CA). A dual-inlet Model Prism VG Isotech (now GV Instruments, Manchester, UK) IRMS system was used.

Solutions Preparation. Dilute Maple Syrup Solution. In a centrifuge tube (50 mL), about 10 g of maple syrup was diluted with 30 mL of water and mixed well.

Artificial Maple Syrup Test Mixture. A total of −66.5 g of cane sugar and 0.24 g of malic acid were dissolved in water and diluted to 250 mL. The concentrations of cane sugar and malic acid correspond to real sample concentrations. To this solution, about 10 drops of artificial maple flavor was added to simulate the color of maple syrup.

Organic Acids Mixture. −A total of 0.01, 0.01, 0.05, 0.10, 0.10, 0.10, and 0.70 g of reagent grade shikimic, fumaric, oxalic, malic, citric, tartaric, and succinic acid, respectively, were dissolved in an LC mobile phase and diluted to 100 mL. To adjust the pH of the solutions, aqueous 14 M NaOH, aqueous 0.5 M NaOH, and aqueous 0.0036 M HCl were used.

Lead Nitrate Solution. A total of −49.7 g of $Pb(NO_3)_2$ was dissolved in water and diluted to 250 mL.

LC Mobile Phase. A total of 13.6 g of KH_2PO_4 was dissolved in water, diluted to 500 mL, and adjusted to pH 2.4 with concentrated H_3PO_4. The solution was filtered through a 0.45 μm filter, type HA (Millipore, Bedford, MA) and degassed.

Sample Selection. Samples were obtained from a wide range of geographical locations. Included were 231 samples from several areas in Quebec, Canada and 26 samples from the United States (Vermont, Maine, New Hampshire, and New York). All the samples were certified to be authentic. The $\delta^{13}C$ analysis of syrup was done to obtain the natural stable carbon isotopic distribution. The distribution normality was tested using the Kolmogorov–Smirnov one-sample test. All statistical analyses were performed using SYSTAT, version 10 (SPSS Science Marketing Department, Chicago, IL). The test showed a normal distribution with $p = 0.982$. The lower and higher values of this distribution were −24.75 and −22.82‰, respectively. Only 13% of the 231 Quebec maple syrup samples were selected to be analyzed for their malic acid stable carbon isotopic ratio. These 30 maple syrup samples were selected to represent the natural distribution of malic acid, which should follow that of the maple syrup, assuming that a correlation exists between these two variables. In addition, all the American maple syrup samples were selected to enhance the area of method application.

USADA 1235

Malic Acid Isolation Procedure and $\delta^{13}C$ Determination. The organic acids contained in the maple syrup were precipated by three consecutive stages. The pH of the dilute maple syrup solution was adjusted to 10.0, and 2 mL of 0.6 M $Pb(NO_3)_2$ was added and mixed well. After 15 min of reaction, the solution was centrifuged at 2500 rotations per minute (rpm) for 10 min. The resulting liquid phase was carefully decanted, the pH was adjusted to 7.0, and 1 mL of 0.6 M $Pb(NO_3)_2$ was added and mixed well. After 15 min of reaction, the solution was centrifuged at 2500 rpm for 10 min. The pH of the decanted liquid phase was adjusted to 7.0, and 0.5 mL of 0.6 M $Pb(NO_3)_2$ was added and mixed well. After 15 min of reaction, the solution was centrifuged at 2500 rpm for 10 min. The liquid phase was discarded. The combined solid residues containing the organic acids were washed 3 times with 10 mL portions of ethanol by agitating for 20 s on a vortex mixer followed by centrifugation at 2500 rpm for 10 min and discarding of the liquid phase. Excess ethanol was removed from the remaining solid residue by being brought to dryness overnight under a fume hood.

The organic acids contained in the solid residue were extracted in four steps. To the residue, 5 mL of the LC mobile phase was added, mixed well, and shaken for 30 min with a lateral mixer. The solution was then centrifuged at 2500 rpm for 10 min, and the liquid phase was decanted and reserved. This procedure was repeated once at 30 min of agitation and twice more with 10 min of agitation. The four extracts containing the organic acids were combined to obtain a volume of about 20 mL.

The pH of this extract was adjusted to 2.0 with concentrated HCl and heated to 90 °C for 30 min. The solution should not be evaporated to dryness to avoid malic acid decarboxylation. After 30 min of reaction, the solution was rapidly cooled in a cold bath (water and ice mixture). Once at room temperature, the pH of the solution was adjusted to 2.4 with 14 M NaOH, and the LC mobile phase was added to obtain a final volume of 20 mL. The solution was now ready for preparative LC separation.

Prior to the injection of the sample into the preparative LC system, all solutions were filtered on a HAWP 0.45 μm filter (Millipore, Bedford, MA). The injection volume was 2 mL delivered by a 2 mL injection loop. The mobile phase flow was set at 9.9 mL min^{-1}. The column eluant was monitored with a UV detector set at 210 nm, and the malic acid retention time was 8.7 min. A home-built automated fraction collector was used to collect the malic acid fraction eluting between 6 and 9.5 min. No isotopic fractionation was noticed (data not shown). The mobile phase of the malic acid fraction was evaporated under a N_2 stream, and the solid residue, containing the malic acid, was dissolved in 0.5 mL of water and transferred quantitatively into a 6 mm i.d. by 9 mm o.d. quartz tube previously annealed at 800 °C. The water was then evaporated under a smooth air jet.

The malic acid solid residue combustion involves the oxidation of the organic compound in a pure oxygen environment at 800 °C. The CO_2 produced was then trapped and purified cryogenically from the other combustion gases (excess O_2 and H_2O) (*23*). The stable carbon isotope ratios were determined using a VG Prism dual-inlet mass spectrometer. All $\delta^{13}C$ values were related to the Vienna Pee Dee Belemnite (VPDB) carbonate standard. The analytical repeatability including the combustion, and the cryogenic distillation step was 0.01‰.

Maple Syrup $\delta^{13}C$ Determination. The $\delta^{13}C$ content of the maple syrup was determined on its dry residue obtained by placing about 7 mg of maple syrup in a vacuum oven at 1×10^{-2} mbar and 65 °C for about 1 h. The dry residue was then transferred into a 4 × 6 mm i.d. and o.d. quartz tube for the combustion and distillation steps.

RESULTS AND DISCUSSION

Reliability of the Malic Acid Isolation Procedure. The $\delta^{13}C$ of commercial malic acid was determined to see if the proposed method induced isotopic discrimination or contamination. Malic acid was purified by preparative LC before determining the $\delta^{13}C$ because fumaric acid was present as an impurity. To isolate malic acid, we increased the recovery time before and after the malic acid peak to avoid the isotopic fractionation that occurs across LC peaks (*22*). By this technique, pure malic acid was collected, and the $\delta^{13}C$ was determined by IRMS to be −29.86 ± 0.02‰ ($n = 7$).

Using this procedure, $\delta^{13}C$ of commercial malic acid isolated from a solution of organic acids was determined to evaluate if the other organic acids present in maple syrup and extracted by lead precipitation could interfere with malic acid. LC separation obtained a complete resolution between each chromatographic peak. Malic acid from this solution was isolated, and $\delta^{13}C$ was determined. The value found was −29.86 ± 0.04‰ ($n = 10$). These results showed no stable carbon isotopic contamination, and since the concentrations used were slightly higher than those found in typical maple syrup samples, with the exception of malic acid, any chromatographic separation with environmental concentrations allows complete malic acid isolation without isotopic contamination.

The complete organic acid separation procedure with lead was tested on the artificial syrup test mixture made from cane sugar, with added malic acid of known $\delta^{13}C$ values and artificial maple flavor. The $\delta^{13}C$ found for isolated malic acid was −29.80 ± 0.07‰ ($n = 9$). The mean value seems to be shifted by 0.06‰ from the original malic acid value. Moreover, the repeatability is higher (0.07 vs 0.04‰). This observation is possible as the analytical repeatability was 0.01‰, which allowed the detection of small isotopic ratio changes occurring by the malic acid isolation procedure.

Three organic sources were suspected to cause the observed behavior: the sucrose, the artificial flavor, and the ethanol used during the washing step. When the sugars were not added into the test maple syrup, the malic acid $\delta^{13}C$ obtained following its isolation was found to be −29.86 ± 0.04‰. Therefore, sucrose seems to be the source of isotopic contamination as the isotopic results show no isotopic shift and the repeatability was reduced to its original value. In fact, a very limited amount of mixing between residual sucrose and malic acid due to overlapping chromatographic peaks could significantly change the malic acid $\delta^{13}C$. To test this hypothesis, a sucrose solution was injected into the preparative LC with the UV detector wavelength set at 190 nm, the maximum absorbance in the UV for carbohydrates. The chromatogram clearly confirmed the occurrence of interference by a sucrose tailing peak within the malic acid fraction. To avoid such an isotopic discrimination caused by sucrose tailing, a hydrolysis step (conversion of sucrose to glucose and fructose) was proposed since glucose and fructose test mixtures did not show any overlapping peaks with malic acid. Sucrose hydrolysis using enzymes, even if the reaction is quantitative and selective, cannot be used because Pb^{2+} is a strong invertase inhibitor (*25*). Acid hydrolysis of sucrose with HCl was found to be the most effective (*26*). Acid hydrolysis tests were then performed before LC separation, and the $\delta^{13}C$ value obtained for malic acid was −29.85 ± 0.05‰ ($n = 11$). By this technique, the overall repeatability matched that reported in the literature by several authors for malic and citric acid isolation in fruit juices, concentrates, and nectars (*17*–*19*).

The overall repeatability was determined by performing 12 independent analyses on the same authentic maple syrup sample. The $\delta^{13}C$ value obtained for malic acid was −26.81 ± 0.08‰ and −24.40 ± 0.04‰ for maple syrup sugars. In the literature, the overall repeatability with $\delta^{13}C$ values varying between 0.1 and 0.5‰ has been reported for equivalent procedures (*17*–*19*). A typical LC chromatogram is shown in **Figure 2**.

$\delta^{13}C$ Values of Sugars and Malic Acid from Authentic Maple Syrup. The stable carbon isotope ratios of sugars and

USADA 1236

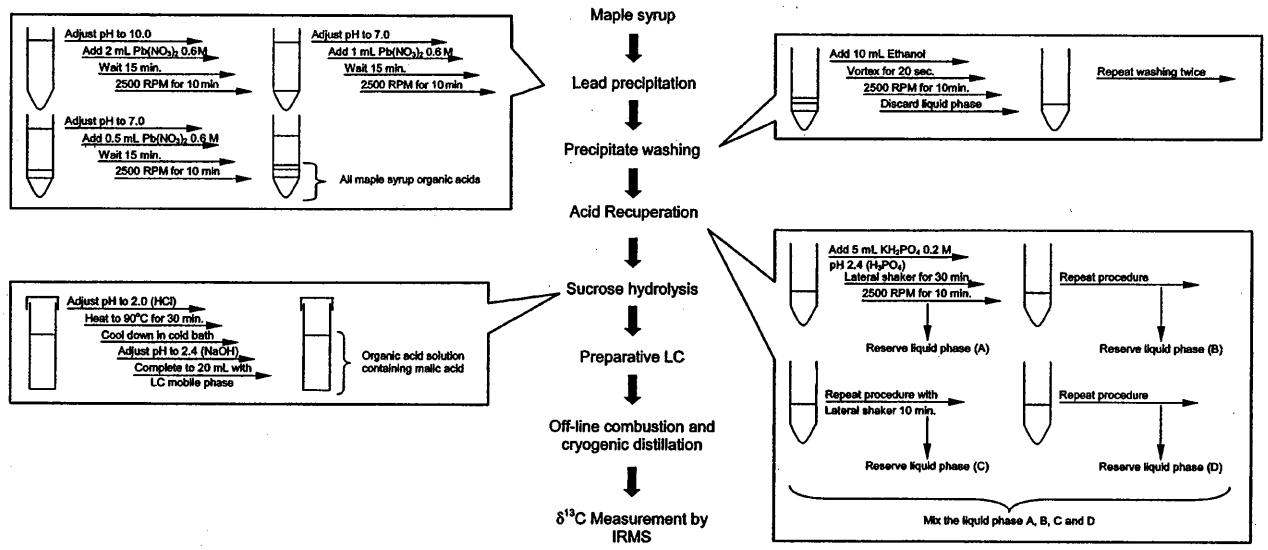


Figure 2. Flow chart of the analytical protocol used for the organic acids isolation from authentic maple syrup (each step is described in the text).

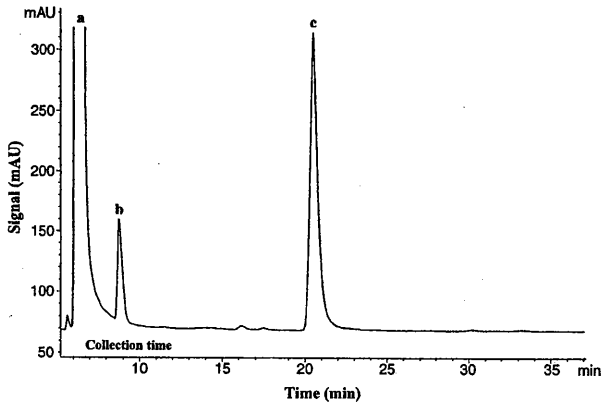


Figure 3. Typical LC chromatogram performed on organic acids extract from authentic maple syrup samples. The chromatographic peaks are (**a**) HNO_3, (**b**) malic acid, and (**c**) fumaric acid.

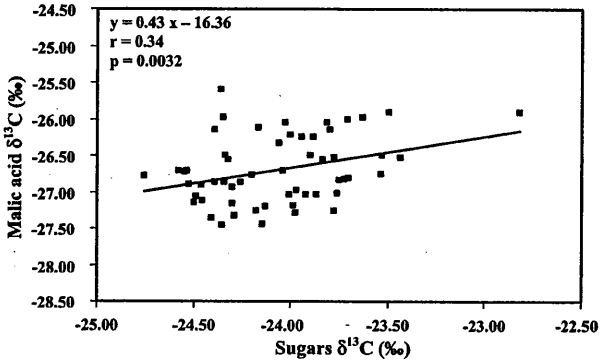


Figure 4. Correlation plot between $\delta^{13}C$ of sugars and malic acid from authentic maple syrup samples.

malic acid of 56 selected authentic maple syrup samples (30 from Quebec, Canada and 26 from the northeastern states) were determined, and the differences between these two values ($\Delta\delta^{13}C = \delta^{13}C_{sugars} - \delta^{13}C_{malic\ acid}$) were calculated from those quantities. The sugar and malic acid isotopic distributions passed the normality Kolmogorov–Smirnov one-sample test with p values of 0.711 and 0.304, respectively. A correlation plot between $\delta^{13}C$ values for sugars and malic acid is presented in **Figure 3**. The regression equation is $0.43x - 16.36$ with $r = 0.34$ ($p = 0.0032$). Higher correlation coefficients are reported in the literature (e.g., 0.75–0.92 between honey and proteins (*11*, *14*), 0.78 and 0.90 between sugars and malic acid in pineapple and apple juices (*17–*, *18*), and 0.89 between sugars and pulp from orange juice (*10*) since juice fabrication involves mechanical treatment only (pressing, centrifugation, and/or filtration)). Maple syrup is produced by the thermal evaporation of the maple sap, a process in which volatile organics evaporate and insoluble organics precipitate and are eliminated by filtration, potentially causing isotope discrimination or fractionation. However, with a p value of 0.0032, the correlation is statistically significant and confirms the isotopic relationship between sugars and malic acid (internal standard). It is surmised that adulteration of a batch of syrup by simultaneous addition of exogenous sugar and malic acid in such a manner to defy detection is sufficiently complex to discourage the practice, and more so if an internal standard is used to improve the detection level (*17–19*).

2-SD and Regression Line, RL, Methods to Detect Adulteration. Typically, the simultaneous $\delta^{13}C$ determination of sugars and the internal standard provides information that allows the threshold limits of adulteration detection to be lower (*19*). As the correlation between the carbon isotope ratios of sugars and malic acid has been established, threshold limits may be determined. Previous studies used a method based on the standard deviation, SD, of the difference of $\delta^{13}C$ values between the sugars and the malic acids, $\Delta\delta^{13}C$, to calculate two fixed detection limits for all samples (*19*). In this study, we propose instead a method based on the actual regression line, RL, existing between $\Delta\delta^{13}C$ and $\delta^{13}C_{malic\ acid}$ to generate detection limits specific to each individual sample. These methods are called the 2-SD and the RL methods, respectively.

In the 2-SD method, the limit values are calculated with a 95% confidence level that allows values within two standard deviations (2-SD) from the mean values of the normal distribution of $\delta^{13}C_{sugars}$ and $\delta^{13}C_{malic\ acid}$ values to be accepted, but lower detection limits are usually obtained when the limits are calculated from $\Delta\delta^{13}C$. Applied to our data, the 2-SD method generated the limits of 1.67 and 3.59‰ from $\Delta\delta^{13}C$. A schematic representation of this method is presented in **Figure 4**, where the two dotted lines represent the 2-SD fixed limits. If a sample $\Delta\delta^{13}C$ value does not lie between these limits, the sample is declared adulterated.

USADA 1237

Table 1. Theoretical Adulteration Limits for Maple Syrup, Honey, and Pineapple Juice Adulterated with Cane, Beet Sugar, or HFCS Using Two Different Internal Standard Methods

	internal standard	adulterant (δ^{13}C ‰)	mean % adulteration 2-SD method (min to max)	mean % adulteration RL method (min to max)	*t*-test (*p* value)
maple syrup	malic acid	cane sugar (−12.3‰)	8.5 ± 3.7 (2–17)	4.9 ± 2.5 (1–10)	2.5×10^{-14}
		Beet sugar (−26.1‰)	48 ± 20 (1–84)	24 ± 12 (1–48)	7.3×10^{-15}
honey	proteins	HFCS[a] (−10.3‰)	10.8 ± 5.2 (4–33)	7.5 ± 4.5 (1–23)	8.0×10^{-15}
	malic acid	beet sugar (−26.1‰)	8.6 ± 3.9 (1–16)	3.7 ± 2.2 (1–8)	2.4×10^{-9}
		HFCS (−10.3‰)	59 ± 32 (8–100)	26 ± 20 (1–100)	3.1×10^{-8}
pineapple juice	Citric acid	beet sugar (−26.1‰)	7.7 ± 3.5 (1–15)	4.8 ± 2.7 (1–10)	6.1×10^{-8}
		HFCS (−10.3‰)	53 ± 27 (5–100)	33 ± 23 (1–100)	2.2×10^{-7}

[a] HFCS: high fructose corn syrup.

In the regression line method (RL method), a plot of the $\Delta\delta^{13}$C values against the $\delta^{13}C_{\text{malic acid}}$ values from authentic maple syrup samples was drawn (**Figure 4**) and demonstrated a good correlation ($r = 0.704$). The least-squares regression line obtained through the data is expressed by $\Delta\delta^{13}C = -0.70\delta^{13}C_{\text{malic acid}} - 16.01$. A virtual space can be obtained by drawing lines parallel to the regression line that encompasses 95% of all the data, being at a vertical distance corresponding to twice the covariance, s_{yx} (*26*). These lines from which $\Delta\delta^{13}$C limits can be calculated have the form

$$\Delta\delta^{13}C(\text{limits}) = -0.70\delta^{13}C_{\text{malic acid}} - 16.01 \pm 2s_{yx}, \text{ where } s_{yx} = 0.25 \quad (1)$$

The covariance term, s_{yx}, has the same statistical meaning as the standard deviation but was applied to both the x and the y correlation values. Therefore, as for the 2-SD method, the calculated limits from twice the covariance, $2s_{yx}$, include 95% (the confidence level) of all point based on a normal distribution of both variables.

By this alternative approach (RL method) consisting of deriving parallel lines on both sides of the regression line, we can calculate for a specific maple syrup sample the two values that establish for $\delta^{13}C_{\text{malic acid}}$ the domain of acceptable $\delta^{13}C_{\text{sugars}}$ values for authenticity. These $\delta^{13}C_{\text{sugars}}$ limit values are expressed from **Figure 4** as

$$\delta^{13}C_{\text{sugars}}(\text{limits}) = \delta^{13}C_{\text{malic acid}} + \Delta\delta^{13}C(\text{limits}) \quad (2)$$

For a reasonable security margin to minimize the error of finding added sugar when there is none, only the highest value of $\delta^{13}C_{\text{sugars}}$(limits) was used to calculate the amount of added C_4 sugars as follows:

$$\%\ \text{add} = \frac{100[\delta^{13}C_{\text{sugars}} - \delta^{13}C_{\text{sugars}}(\text{limits})]}{\delta^{13}C_{\text{adulterant}} - \delta^{13}C_{\text{sugars}}} \quad (3)$$

Improved Detection of Addition of a C_4 and C_3 Product in Maple Syrup Using the RL Methods. Adulteration was mathematically simulated for the set of 56 maple syrup samples. The effect of adding exogenous C_4 (beet) and C_3(cane) sugars, respectively, with δ^{13}C values of −12.3 and −26.1‰ were calculated by the following equation:

$$\delta^{13}C_{\text{aldulterated}} = \frac{(\delta^{13}C_{\text{add}}\ \%\ \text{add}) + [\delta^{13}C_{\text{initial}}(100(100 - \%\ \text{add}))]}{100} \quad (4)$$

where % add is the amount of C_4 and C_3 product added, $\delta^{13}C_{\text{add}}$ is the δ^{13}C value of the C_4 and C_3 product added, and $\delta^{13}C_{\text{initial}}$ is the initial δ^{13}C value of the maple syrup sugars.

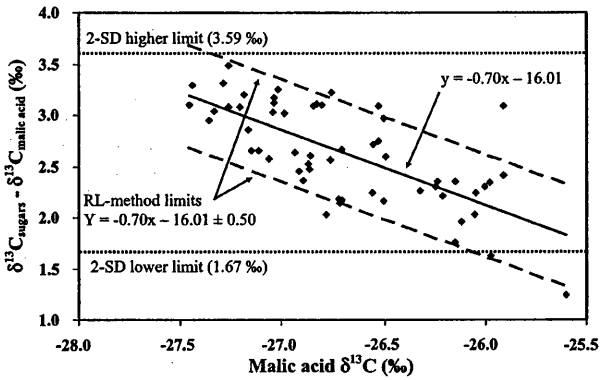


Figure 5. Correlation plot between δ^{13}C of malic acid and the isotopic ratio difference ($\delta^{13}C_{\text{sugars}} - \delta^{13}C_{\text{malic acid}}$) from authentic maple syrup samples. Upper and lower limits for the 2-SD and the RL method at 95% confidence level are drawn on the graph.

To set the expected adulteration detection limits for both methods, the $\delta^{13}C_{\text{aldulterated}}$ was increased until its value was outside the domain of acceptable values for authenticity. For the RL method, eqs 1 and 2 were used to calculate each maple syrup limit, and for the 2-SD method, the fixed limit $\Delta\delta^{13}$C values of 1.67 and 3.59‰ (from $2.63 \pm 2 \times 0.48$‰) were used. The mean threshold adulteration detection limits for a C_4 sugar was found to be 4.9 ± 2.5% (range of adulteration from 1 to 10%) calculated from the RL method and 8.5 ± 3.7% (range of adulteration from 2 to 17%) calculated from the 2-SD method. A paired *t*-test ($p = 2.5$ E-14) confirmed that the RL-method gives a lower adulteration detection limit of 3.6 ± 2.7% as compared to the 2-SD method (**Table 1**). The RL-method also gives lower adulteration limits for C_3-sugars adulteration than the 2-SD method, 48 ± 20% with the 2-SD method in comparison with 24 ± 12% with the RL-method (p from paired *t*-test is 7.3×10^{-15}).

In **Figure 4**, two samples may be seen as outliers: sample A, $\delta^{13}C_{\text{sugars}}$ −24.36‰, $\delta^{13}C_{\text{malic acid}}$ − 25.60‰ and sample B, $\delta^{13}C_{\text{sugars}}$ −22.82‰, $\delta^{13}C_{\text{malic acid}}$ −25.91‰. According to the 2-SD method, sample A will be identified as adulterated ($\delta^{13}C_{\text{malic acid}}$ −25.60‰, $\Delta\delta^{13}$C 1.24‰) but will be identified as authentic by the RL method (**Figure 5**). Contrary, sample B will be identified as adulterated by the RL method ($\delta^{13}C_{\text{malic acid}}$ −25.91‰, $\Delta\delta^{13}$C 3.09‰) and authentic by the 2-SD method (**Figure 5**). As all maple syrup samples used in this study were certified as authentic by the producers, this observation brings to light the limitations of both methods (SD and RL). The statistics were performed at a confidence level of 95%, which opens the door to false positive interpretations. Regulatory bodies would use such results as an indicator of adulteration and proceed with additional confirmation analyses before making a final adulteration statement on a specific sample. Adulteration statements should never be based on a single

adulteration detection method as demonstrated by **Figure 5**. Higher confidence levels have been reported for honey adulteration where a 4-SD method was used to achieve a probability of error as low as 1 in 25 000 (*15*). This approach is unusual and seems specific to honey adulteration where for other foodstuffs, a confidence level of 95% is well accepted by the scientific community.

Improvement on Other Food Products Adulteration with the RL Methods. The potential application of this new technique to the adulteration of other food products was evaluated. We have used the isotopic data for honey (50 samples) published by White and Winters (*11*) where the proteins of honey were employed as internal standard and those from Jamin et al. (*17*) for pineapple juices (30 samples) where malic and citric acids were used as internal standards. The simulated adulteration involved the addition of high fructose corn syrup (HFCS $\delta^{13}C = -10.3‰$) to honey and HFCS and beet sugar to pineapple juices. The calculated adulteration limits are reported in **Table 1**. For all cases, the adulteration limit of the RL method was statistically better than the 2-SD method for honey adulteration. In the case of pineapple juices where malic acid was used as internal standard, the 2-SD method was better for one sample for beet sugar adulteration and two samples for HFCS adulteration. When citric acid was used, the 2-SD method was better for two samples either for beet sugar or HFCS adulteration. With these results, the RL technique can be applied to several foodstuffs where the technique of the internal standard is applied to detect adulteration, and most of the time, this technique is better to detect sugar adulteration than the 2-SD method.

C_3- versus C_4-Plants as Adulterant. We also observe that the RL method is especially powerful in the cases where the $\delta^{13}C$ values of the adulterant and the adulterated food are similar. In fact, an improvement of 24 ± 17% with values as high as 58% when beet sugar (C_3-plant, −26.1‰) was used in comparison to an improvement of 3.6 ± 2.7% when cane sugar (C_4-plant, −12.3) was used to simulate adulteration on maple syrup (C_3-plant) was observed (**Table 1**). The same trend was observed for adulteration simulation on pineapple juices (C_4-plant) with malic acid as internal standard where an improvement of 4.9 ± 3.1% was found when beet sugar was added as compared with 33 ± 24% when HFCS (C_4-plant) was added. With citric acid as an internal standard, the improvement for beet sugar detection was 2.8 ± 2.1% in comparison with 20 ± 16% for HFCS detection.

The $\delta^{13}C$ of maple syrup sugars and malic acid has been determined on 56 authentic maple syrup samples from Canada and the United States. The mean $\delta^{13}C$ of maple syrup sugars found is −24.07‰, and the mean $\delta^{13}C$ of malic acid found is −26.71‰, which corresponds to typical C_3-plant stable carbon isotopic ratios. Organic acids were isolated from maple syrup by lead precipitation using an optimized sample preparation procedure, and pure malic acid was separated from the other organic acids by preparative reversed-phase liquid chromatography. Good correlation between sugars and malic acid has been found ($r = 0.34$, $p = 0.0032$), defining malic acid as a suitable internal standard. The technique has shown no isotopic discrimination for sugars or malic acid $\delta^{13}C$ determination.

To improve the actual decision limit of maple syrup adulteration, an improved calculation technique was developed. This technique is based on the correlation existing between malic acid, the internal standard, and the isotopic difference between maple syrup sugars and malic acid ($\Delta\delta^{13}C = \delta^{13}C_{sugars} - \delta^{13}C_{malic\ acid}$). The regression line is used to determine for each individual maple syrup sample the authentic $\delta^{13}C_{sugars}$ range derived from the malic acid $\delta^{13}C$.

To validate this technique, simulated sugar spiking has been performed with cane (C_4-plant, $\delta^{13}C$ −12.3‰) or beet (C_3-plant, $\delta^{13}C = -26.1‰$) on the 56 authentic maple syrup samples, and the theoretical adulteration limit has been evaluated for both the RL and the 2-SD methods. A narrower detection limit of 3.6% is obtained for cane sugar adulteration and 24.6% for beet sugar adulteration with the RL method, which has also been applied to honey and pineapple juice adulteration by using data found in the literature. In those cases, beet sugar and HFCS (C_4-plant, $\delta^{13}C$ −10.3‰) were the target adulterants. In both cases, the RL method provided a narrower detection limit, making it suitable for use in food adulteration. This technique is all the more suitable when the photosynthetic origin of the food product and the adulterant is the same as shown by the results obtained for maple syrup adulteration by beet sugar and for pineapple juice adulteration by HFCS.

SAFETY

Lead nitrate is highly toxic if ingested and/or inhaled, and care must be exercised by handling in a chemical fume hood using adequate personal safety equipment such as impervious gloves, apron, or protective clothing. Lead nitrate should be stored in a cool, well-ventilated area (lower than 30 °C), away from sparks and flames. Waste lead material should be disposed of in accordance with applicable local, state, and federal regulations.

ACKNOWLEDGMENT

We thank Luidgi Morais, Michel Boutin, and David Gauthier for their technical support during the experiments, and we gratefully acknowledge the many comments and proofreading of the manuscript by Gilles Paillard.

Supporting Information Available: Stable carbon isotope ratios of sugars and malic acid from authentic maple syrup samples. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Carro, O.; Hillaire-Marcel, C.; Gagnon, M. Detection of adulterated maple products by stable carbon isotope ratio. *J.—Assoc. Off. Anal. Chem.* **1980**, *63*, 840−844.

(2) Morselli, M. F.; Baggett. L. K. Mass spectrometric metermination of cane sugar and corn syrup in maple syrup by use of $^{13}C/^{12}C$ ratio: Collaborative study. *J.—Assoc. Off. Anal. Chem.* **1984**, *67*, 22−24.

(3) Martin, G. G.; Hanote, V.; Less, M.; Martin, Y. L. Interpretation of combined H^2 SNIF/NMR and C^{13} SIRA/MS analyses of fruit juices to detect added sugar. *J.—Assoc. Off. Anal. Chem.* **1996**, *79*, 62−72.

(4) Hillaire-Marcel, C.; Carro-Jost, C.; Jacob, C. Composition isotopique C^{13}/C^{12} du saccharose et du glucose de diverses origines et contrôle de l'authenticité des sirops et sucres d'érable. *J. Inst. Can. Sci. Technol. Aliment.* **1977**, *10*, 333−335.

(5) Martin, Y. L. Detection of added beet or cane sugar in maple syrup by the SNIF-NMR method: collaborative study. *J. AOAC Int.* **2001**, *84*, 1511−1521.

(6) Calvin, M.; Bassham, J. A. In *The Photosynthesis of Carbon Compounds*; Calvin, M., Bassham, J. A., Eds.; Benjamin: New York, 1964.

(7) Hatch, M. D.; Slack, R. C.; Jonhson, H. S. Further studies on a new pathway of photosynthetic carbon dioxide fixation in sugar cane and its occurrence in other plant species. *Biochem. J.* **1967**, *10*, 417−422.

USADA 1239

(8) Lerman, J. C.; Queiroz, O. Carbon fixation and isotope discrimination by a crassulacean plant: dependence on the photoperiod. *Science* **1971**, *183*, 1207−1209.
(9) Moore, R.; Clark, W. D.; Vodopich, D. S. In *Botany*; Moore, R., Clark, W. D., Vodopich, D. S., Eds.; WCB/McGraw-Hill: Boston, MA, 1998.
(10) Bricout, J.; Koziet, J. Control of the authenticity of orange juice by isotopic analysis. *J. Agric. Food Chem.* **1987**, *35*, 758−760.
(11) White, W. J.; Winters, K. Honey protein as internal standard for stable carbon isotope ratio detection of adulteration of honey. *J.—Assoc. Off. Anal. Chem.* **1989**, *72*, 907−977.
(12) Jamin, E.; González, J.; Bengoechea, I.; Kerneur, G.; Iriondo, C.; Martin, G. G. Proteins as intermolecular isotope reference for detection of adulteration of fruit juices. *J. Agric. Food Chem.* **1998**, *46*, 5118−5123.
(13) Lee, H. S.; Wrolstad, R. E. Stable isotopic carbon composition of apples and their subfractions juice, seeds, sugars, and nonvolatile acids. *J.—Assoc. Off. Anal. Chem.* **1988**, *71*, 795−797.
(14) White, W. J. Internal standard stable carbon isotope ratio method for determination of C_4-plant sugars in honey: collaborative study and evaluation of improved protein preparation procedure. *J. AOAC Int.* **1992**, *75*, 543−548.
(15) White, W. J.; Winters, K.; Martin, P.; Rossmann, A. Stable carbon isotope ratio analysis of honey: validation of internal standard procedure for worldwide application. *J. AOAC Int.* **1998**, *81*, 610−619.
(16) Jamin, E.; González, J.; Bengoechea, I.; Kerneur, G.; Remaud, G.; Iriondo, C.; Martin, G. G. Proteins as intermolecular isotope reference for detection of adulteration of fruit juices. *J. Agric. Food Chem.* **1998**, *46*, 5118−5123.
(17) Jamin, E.; González, J.; Remaud, G.; Naulet, N.; Martin, G. G. Detection of exogenous sugars or organic acids addition in pineapple juices and concentrates by C^{13} IRMS analysis. *J. Agric. Food Chem.* **1997**, *45*, 3961−3967.
(18) Jamin, E.; González, J.; Remaud, G.; Naulet, N.; Martin, G. G.; Weber, D.; Rossmann, A.; Schmidt, H.-L. Improved detection of sugar addition to apple juices and concentrates using internal standard ^{13}C IRMS. *Anal. Chim. Acta.* **1997**, *347*, 359−368.
(19) Jamin, E.; González, J.; Bengoechea, I.; Kerneur, G.; Remaud, G.; Naulet, N.; Martin, G. G. Measurement of $^{13}C/^{12}C$ ratios of sugars, malic acid, and citric acid as authenticity probes of citrus juices and concentrates. *J. AOAC Int.* **1998**, *81*, 604−609.
(20) Stuckel, J. G.; Low, N. H. The chemical composition of 80 pure maple syrup samples produced in North America. *Food Res. Int.* **1996**, *29*, 373−379.
(21) Mattiuz, E. L.; Perone, R. A. Analysis of trace levels of organic acids in nutritive sweeteners. *LC-GC* **1986**, *4*, 552−562.
(22) Gensler, M.; Schmidt, H.-L. Isolation of the main organic acids from fruit juices and nectars for carbon isotope ratio measurements. *Anal. Chim. Acta* **1994**, *299*, 231−237.
(23) AOAC official method 959.13. *Malic Acid in Maple Syrup*, 17th ed.; AOAC Intl.: Gaithersburg, MD, 2000.
(24) Michaud, D.; Morais, L.; Cha, L.; Baril, M.; Paquin, R. *42nd Annual Conference on Mass Spectrometry and Allied Topics*; American Society for Mass Spectrometry: Chicago, IL, 1994; p 834.
(25) Pancoast, H. M.; Junk, W. R. In *Handbook of Sugars for Processors, Chemists, and Technologists*; Pancoast, H. M., Junk, W. R., Eds.; AVI Publ. Co.: Westport, CT, 1973.
(26) Myrbäck, K. In *The Enzymes*; Boyer, P. D., Lard, H., Myrbäck, K., Eds.; Academic Press: New York, 1959; pp 379−395.
(27) Alder, H. L.; Roessler, E. B. In *Introduction to Probability and Statistics*; Alder, H. L., Roessler, E. B., Eds.; W. H. Freeman and Co.: San Francisco, CA, 1972; pp 206−210.

Received for review August 22, 2006. Revised manuscript received November 27, 2006. Accepted November 27, 2006. This research was supported by Université Laval, Agriculture and Agrifood Canada, and the Natural Science and Engineering Research Council of Canada (Grant STR0167418).

JF062413A

ELSEVIER

Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols

Cedric H.L. Shackleton,* Andy Phillips,† Tony Chang,* and Ye Li‡

**Children's Hospital Oakland Research Institute, Oakland, California, USA; †Micromass UK Limited, Manchester, England, UK; and, ‡National Research Institute of Sports Medicine, Beijing, China*

A gas chromatographic combustion isotope ratio mass spectrometric (GC/C/IRMS) method was used for studying the incorporation of exogenous testosterone enanthate into excreted urinary 5α- and 5β-androstane-3α,17β-diols. A multistep but straightforward work-up procedure produced a simple GC chromatogram of urinary steroid acetates composed principally of two androstanediols and pregnanediol. It is anticipated that such a method may form the basis of a doping control test for testosterone that could be used as a primary method during major sporting events or alternatively as a verification technique. Urine samples from five individuals were collected before and after administration of testosterone enanthate (250 mg). The $\delta^{13}C$ °/00 value of androstanediols was around −26 to −28 during the baseline period and decreased to about −29 to −30 in the days following synthetic testosterone administration. One of the other major steroids in the chromatogram, pregnanediol, was utilized as the "internal standard," because its $\delta^{13}C$°/00 values did not markedly change following testosterone administration, remaining at −25 to −27. In all subjects studied, the $\delta^{13}C$°/00 values for androstanediols were reduced sufficiently over 8 days to confirm administration of synthetic testosterone. Although steroids isolated from urine of normal individuals from 12 different countries gave values between −24 and −28, this seemed not to be related to nationality or region. The most likely variable is the proportion of plants with low and high carbon 13 content in the diet. This variable is likely to be more affected by individual food preferences than broad ethnic food divisions. In this paper, we propose a ratio of $\delta^{13}C$°/00 for androstanediols to pregnanediol as a useful discriminant of testosterone misuse, a value above 1.1:1.0 being indicative of such misuse. The work-up procedure was designed for batch analysis and to use only simple techniques, rather than employ further instrumentation, such as high-performance liquid chromatography (HPLC), in purifying steroids for GC/C/IRMS. (Steroids **62**:379–387, 1997) *© 1997 by Elsevier Science Inc.*

Keywords: isotope ratio mass spectrometry (IRMS); doping control; testosterone

Introduction

The increasing need in sport for proving testosterone misuse requires new methodologies. For many years, a testosterone/epitestosterone (T/E) excretion ratio determined by gas chromatography–mass spectroscopy (GC–MS) of greater than 6:1 has been used as the hallmark for confirmation of drug administration,[1] but this method is fallible. For one thing, occasional drug-free individuals give a ratio >6, and a high ratio can also be adjusted downward by simultaneous administration of epitestosterone. We have found that in eight Chinese subjects given 250 mg testosterone enanthate, only three gave T/E values >6 on more than 1 day, demonstrating a high rate of false negatives, at least in this racial group.

In 1990, Southan and co-workers[2] used isotope ratio mass spectrometry (IRMS) to show that synthetic testosterone had a different ^{13}C content than endogenous hormone. This is a reflection of the origin of the materials, because all testosterone, both endogenous and synthetic, is ultimately of plant origin. Gonadal testosterone is made from precursor molecules derived from a wide variety of vegetable mate-

Address reprint requests to Cedric H.L. Shackleton, Children's Hospital Oakland Research Institute, 747 52nd Street, Oakland, CA 94609, USA.
Received July 25, 1996; accepted November 4, 1996.

Steroids 62:379–387, 1997
© 1997 by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0039-128X/97/$17.00
PII S0039-128X(96)00253-X

rials eaten by humans or by the animals humans eat. Individual plants discriminate to different degrees against $^{13}CO_2$, and particular species are known to have high or low levels of ^{13}C in their biomolecules.[3] In human bodies, the ^{13}C content, therefore, reflects an average of the ^{13}C content of all the plant material eaten by humans and our animal protein providers. Synthetic testosterone, in contrast, is generally made from a single plant species, mostly soy, so the ^{13}C content has a defined value reflecting the relatively low ^{13}C content of this plant. Thus, a significant difference in ^{13}C content between gonadal and soy testosterone could provide the basis for developing a definitive test for hormone misuse. Differences in carbon isotope ratios referred to by the symbol δ, defined as the difference in isotope ratio between the sample and an international carbonate standard "PDB." Although this is the accepted standard, for common usage a calibrated international standard of CO_2 is used. The values reported for δ carbon isotope ratios are

$$\delta^{13}C^{0}/_{00} = \left(\frac{\text{Ratio sample} - \text{Ratio PDB}}{\text{Ratio PDP}}\right) \times 1000$$

Becchi and coinvestigators have published pioneering studies on development of a method employing IRMS for determination of carbon isotope ratio of testosterone extracted from urine.[4,5] They demonstrated that, providing sufficient urine was available, the endogenous or exogenous origin of testosterone could be readily determined. A major remaining problem demonstrated by these studies relates to sensitivity of the analysis, because the quantity of urine collected from athletes is relatively small (about 75 mL), and this is divided into two, a primary (A) and a secondary sample (B). On each sample, nonsteroidal drug metabolites must be analyzed, as well as anabolic steroid screening and measurement of the T/E ratio.

We have attempted to improve the methodology to allow more sensitive analysis. It was decided to forgo any attempt to analyze testosterone itself and to concentrate on analysis of its metabolites 5α-androstane-3α,17β-diol (5αAD) and 5β-androstane-3α,17β-diol (5βAD). Our overall objective was to easily produce a single sample for analysis containing a few defined steroids to include the androstanediols and steroids we call "endogenous reference compounds" (ERCs). An ERC, in this instance, is a steroid whose carbon isotope ratio could not be altered through administration of exogenous testosterone. Aguilera, Becchi, and co-workers in their most recent publication use cholesterol and 5-androstene-3β,17β-diol as ERC.[5] To achieve our overall objective, we developed simple methodology adaptable to batch analysis, which required no liquid chromatographic (HPLC) instrumentation. Using this methodology, we determined the $\delta^{13}C^{0}/00$ of the androstanediols present in urine following administration of testosterone enanthate to five volunteers. This communication presents the results of this study.

Experimental

Materials

Testosterone enanthate, Testoviron depot® was obtained from Schering, Japan. Reference steroids were obtained from Sigma (St. Louis, Missouri, USA), which was also the supplier of Girard reagent T (carboxymethyl, trimethyl ammonium chloride hydrazide) and sodium bismuthate. Sephadex LH 20 was a product of Pharmacia AB and Sep-pak® cartridges, a product of Waters Corp. (Milford, Massachusetts, USA). β-glucuronidase/aryl sulfatase was obtained from Sigma (Type H1) and Boehringer Mannheim (Mannheim, Germany). Solvents were of analytical grade.

Individuals studied

Eight Chinese male subjects aged 19–22 were studied, although GC/C/IRMS analysis was only conducted on five. Permission for undertaking these experiments was obtained from the Chinese National Research Institute of Sports Medicine, and consent was obtained from the participants. Spot morning urine samples were collected for 2 days prior to an intramuscular injection of 250 mg testosterone enanthate. Two urine samples (0–8 h and 8–24 h) were collected for the first 4 days after administration, although only aliquots of the early morning sample were subject to analysis. Morning spot urine samples were collected on the 5–9th days after administration and on days 11, 13, and 15.

Determination of testosterone/epitestosterone ratio (T/E)

Urinary testosterone and epitestosterone were quantified in all the samples using an adaptation of the method of Donike et al.[6] These measurements were carried out by one of us (YL) in China at the National Institute of Sports Medicine. T/E ratios were then determined.

Preparation of steroid extract for GC/C/IRMS

A flowsheet summarizing the methodology is shown in Figure 1. Urine (typically 25 mL) was extracted by Sep-pak® cartridge according to the method of Shackleton and Whitney.[7] Once dried, the extract was dissolved in 3 mL 0.1 M acetate buffer pH 5 and *Helix pomatia*-derived β-glucuronidase/sulfatase (12 mg Sigma

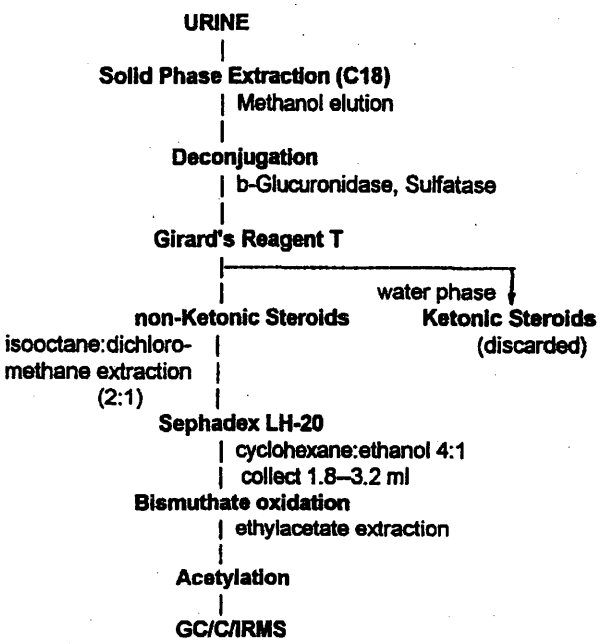


Figure 1 Flow sheet of the urinary extraction method.

USADA 1242

type H1 powder, 100 μL Boehringer liquid enzyme) was added. Hydrolysis was allowed to proceed for 3 h at 55°C. A Girard separation was carried out to separate carbonyl-containing steroids (ketonic) from noncarbonyl-containing (nonketonic) steroids.[8,9] To the hydrolyzed urine mixture, 2 mL glacial acetic acid and 100 mg Girard reagent T was added. The solutions were placed in an oven at 100°C for 30 min. The nonketonic steroids were extracted by 2 × 5 ml isooctane: dichloromethane 2:1 (v/v), and the solvent was dried. Small columns of 0.5 Sephadex LH-20 were prepared in Pasteur pipettes, the Sephadex being allowed to swell in the cyclohexane: ethanol (4:1) solvent system before preparation.[10] The steroid extract dissolved in 100 μL of the same solvent mixture was added to these columns. Solvent eluting between 1.8 and 3.2 mL was collected and dried. Acetic acid (0.1 mL), water (0.1 mL), and 5 mg sodium bismuthate were added.[9] Oxidation was allowed to proceed for 2 h, and after neutralization (0.5 mL of 0.5 M acetate buffer), the mixture was extracted with 4 mL ethyl acetate. After drying, steroid acetates were prepared overnight with 50 μL acetic anhydride and 50 μL pyridine. The acetates were analyzed by GC/C/IRMS.

Gas chromatography combustion isotope mass spectrometry (GC/C/IRMS)

A schematic representation of the GC/C/IRMS instrumentation is illustrated in Figure 2. The acetylated steroid samples were kept refrigerated until analysis. Cyclohexane (20 μL; 99.9% pure from Sigma Chemicals 27-0625-8) was added to each vial, and one-tenth (2 μL) of each sample was injected splitless onto a J&W 30 m DB17 capillary column housed in a Fisons 8000 series GC. The injector was kept at 220°C. The temperature program was as follows: starting temperature 50°C (1 min), followed by rapid temperature increase (25° min) to 300°C, where it was held for 15 min.

The separated components were heart-split into the combustion furnace filled with copper oxide wires (Elemental Microanalysis Limited, UK) held at 850°C. The combustion gases were passed through a nafion membrane water removal trap, and the remaining CO_2 was analyzed on a Micromass isochrom isotope mass spectrometer. The mass spectrometer consisted of an electron impact source running at 400 μA trap current, the ionized CO_2 gas, was focused by a magnet onto three Faraday collectors. The ions collected were those at masses 44, 45, and 46. The Micromass data system calculated the areas of the beams and subtracted any background; whereupon, calculation of the ^{13}C δ values for the successive peaks were carried out.

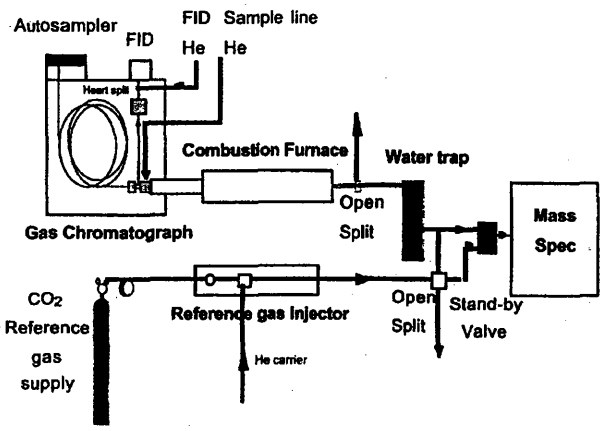


Figure 2 Schematic of the Micromass GC/C/IRMS instrument.

Results and discussion

Evaluation of gas chromatographic columns

The objective of the study was to measure $\delta^{13}C^0/00$ for urinary androstanediols formed as metabolites of testosterone. In our initial studies (Subject 1), we undertook chromatography on DB1 capillary columns but did not get resolution of the two diacetylated epimers (Figure 3A). Later employment of a DB17 column (Figure 3B) allowed separation of the epimers as well as the ERC, pregnanediol diacetate, and pregnanetriol diacetate (Figure 3B).

Confirmation of identity of steroids in extracts

Prior to sending the first samples for GC/C/IRMS analyses in England, the identities of the principal components of the chromatogram were confirmed by GC/MS. This was carried out on a Hewlett-Packard 5970 instrument housing a 15 meter DB1 capillary column. The peaks chosen for GC/C/IRMS analyses had retention times and electron impact mass spectra identical to those of 5α- and 5β-androstanediol diacetate and pregnanediol diacetate. Reference steroids for these compounds were also analyzed on the GC/C/IRMS instrument using both DB1 and DB17 columns, and these gave identical retention times to the urinary steroids. Pregnanetriol could also be analyzed by GC/C/IRMS.

Achievement of work-up procedure objective

The method developed and utilized had the following qualities. First, the Girard separation almost completely removed carbonyl containing steroids from the hydrolyzed extracts, which probably represent 75% of urinary steroids. Exceptions may be the 11-carbonyl containing steroids that probably do not react because of the hindered nature of that functional group. Second, a crude micro Sephadex LH-20 column separation effectively produced a fraction containing steroids with two and three functional groups. Third, sodium bismuthate oxidation was designed into the procedure as a means of removing remaining long-retention time pregnane metabolites, thus allowing shorter periods between injection. Many of the quantitatively more important metabolites are converted into 17-oxygenated C_{19} steroids by the procedure. Fourth, acetylation provided steroids with good gas chromatographic properties that were readily separated. Fifth, despite the complexity of the steroid fraction of urine, the final chromatogram was simple and composed of only a few peaks for which $\delta^{13}C^0/00$ could be determined with accuracy.

$\delta^{13}C^0/00$ value of the synthetic testosterone

Testosterone acetate prepared from the Japanese testosterone enanthate used for injection in these studies gave a $^{13}C^0/00$ value of −30.41, a value close to the lowest value obtained for androstanediol diacetate measurements obtained in the subjects studied following testosterone administration.

We also analyzed five other current products and one synthetic sample made more than 40 years ago. The following results were obtained: testosterone of Chinese manufacture −30.40; U.S. manufacture −30.38; two Czech products,

USADA 1243

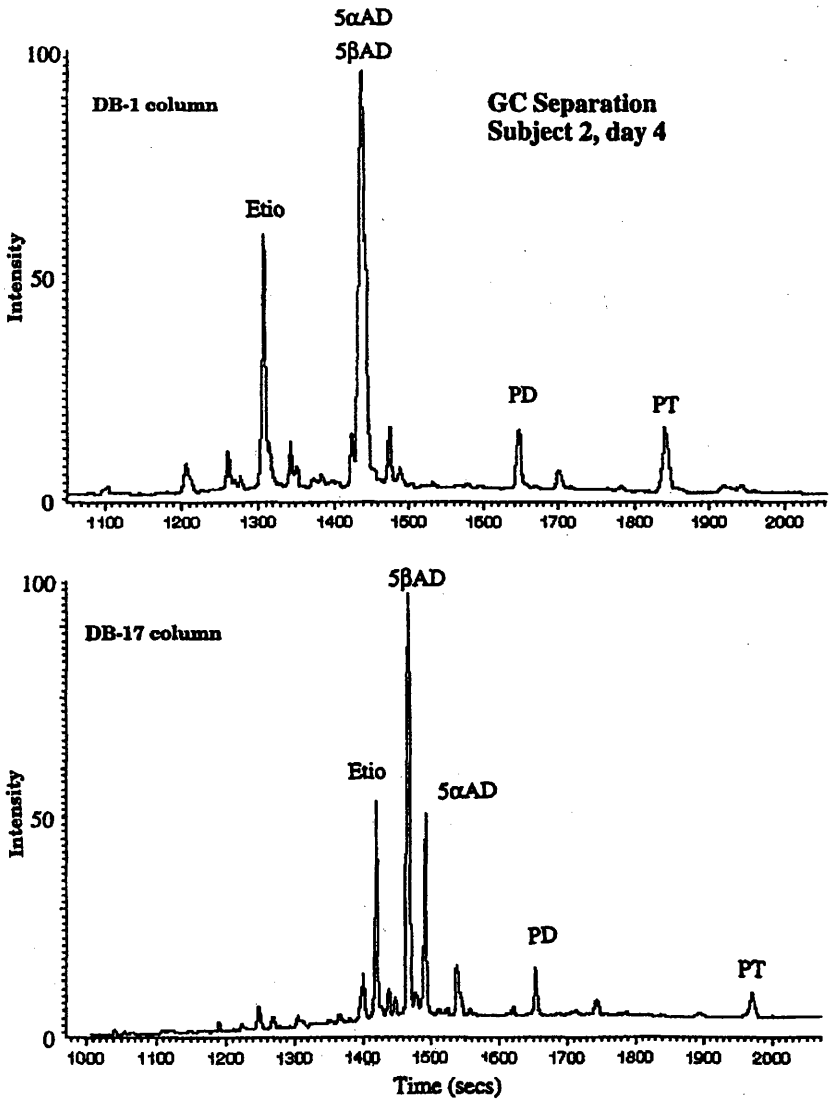


Figure 3 Gas chromatographic separation (on GC/C/IRMS instrument) of acetylated urinary steroid fraction. This sample was initially separated on a DB1 column, which failed to resolve the 5α- and 5β-androstanediols (upper panel). Later employment of a DB17 column permitted individual measurement of the epimers (lower panel). Both columns also resolved etiocholanolone, produced by bismuthate oxidation of pregnanetriol, pregnanediol, and nonoxidized pregnanetriol.

−29.28 and −29.15, respectively; and one Russian −30.22. The U.S. sample of more than 40 years old gave a value of −33.18, probably reflecting the different plant origin of synthetic steroids made long ago. We believe that testosterone is currently made mostly from soy by-products, although originally sterol constituents of the Mexican yam were the primary precursors.

Results for individual subjects

With the exception of Subject 1, a DB17 capillary column was utilized that allowed separate analysis of 5α- and 5-β androstanediols. Graphs illustrating the $\delta^{13}C^{0}/00$ results are shown in Figure 4A–F. In all cases, the $\delta^{13}C^{0}/00$ values for the androstanediols fell significantly following testosterone administration.

Figure 4 illustrates two other features. One is the adequacy of making single measurements of $\delta^{13}C^{0}/00$ for each sample. Panel B and C show results obtained by plotting the average of duplicate measurements (B) and of the *first* measurement of the duplicate pair (C). Essentially these graphs are identical, suggesting duplicate GC/C/IRMS analyses of the same preparative extract are unnecessary. The second feature relates to the trend of slightly increasing δ values for pregnanediol during the study period of Subject 3 (Figure 4D). Because this is not caused by random irreproducibility we proposed that the individual changed his diet substantially to foodstuffs with higher $\delta^{13}C^{0}/00$ values.

USADA 1244

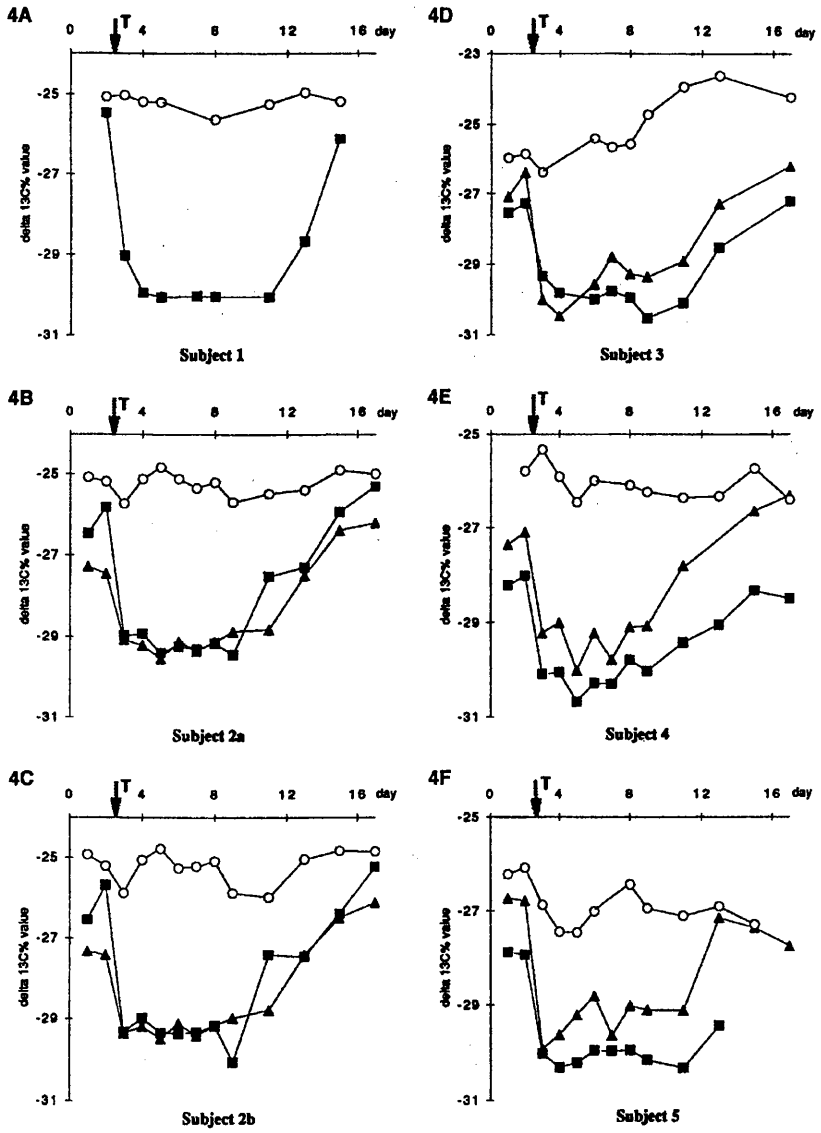


Figure 4 Values of $\delta^{13}C^{0}/00$ for all five subjects throughout the study period. The open circles represent pregnanediol, the closed rectangles 5βAD and closed triangles 5αAD. 4A shows the values obtained for Subject 1 in which only one baseline sample was analyzed, the testosterone being administered on the 2nd day of study. Both androstanediols were measured together on a DB1 column, 4B shows plots for Subject 2 obtained by averaging values for the duplicate runs, and 4C shows equivalent plots through only using the first analysis. 4C, 4D, and 4E are plots of Subjects 3, 4, and 5. For Subjects 2–5 the testosterone was administered after the second baseline sample (day 3 of study).

Designing a method for universal use demands final agreement of numerative values above and below which individuals are considered to have or to have not illicitly used a drug. In our studies with the Chinese subjects, it can be stated that for the five individuals, none had androstanediol $\delta^{13}C^{0}/00$ values less than −28.3 during the control period, and establishing a conservative cut-off value of −29.0 would clearly pick up all drug users for about 7 days after administration. Additionally, we considered that measurement of analyte/ECR ratios could allow numerative values to be established that are to some degree independent of variables introduced through using the methodology in different laboratories with different instrumentation. In Figure 5A, we show the $\delta^{13}C^{0}/00$ ratio of combined androstanediols (average of 5α- and 5β-) to pregnanediol (PD) for the five individuals studied so far. All baseline ratios fell below 1.08, and from these data, we would suggest that a ratio of greater than 1.1 could be used to confirm testosterone administration. When we averaged the data for the five individuals, we found that the AD/PD ratio value of 1.1 was exceeded for 11 days (Figure 5B). Because a combined androstanediol measurement is carried out in the Aguilera et al. studies,[5] comparable data could be prepared from their measurements, although in their case, cholesterol would be the ERC used for ratio determination.

USADA 1245

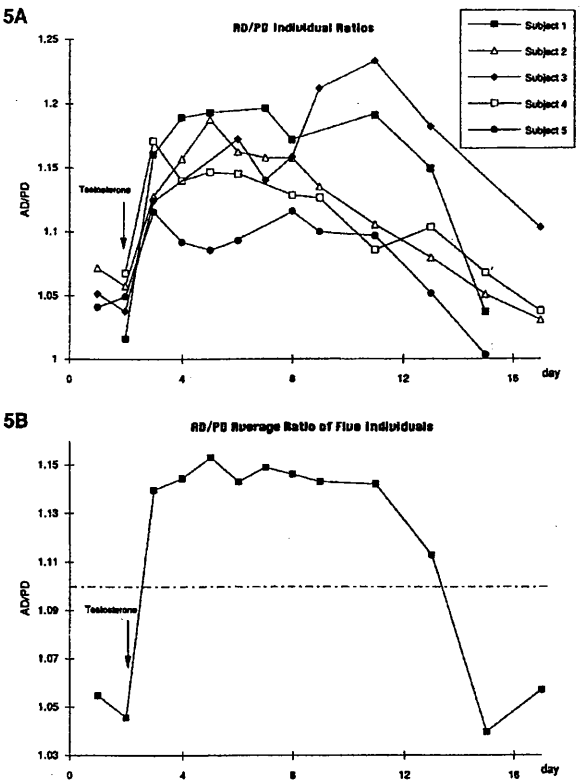


Figure 5 Androstanediol/pregnanediol ratio. A) ratios of $\delta^{13}C^0/00$ for the androstanediols (mean of 5α and β) to pregnanediol for the five subjects; B) average of the ratio for the five subjects for each day; the dotted line represents the proposed discriminant value (1.10) for confirmation of testosterone administration.

Reproducibility of isotope measurements and accuracy

For the five subjects studied, ideally 26 or more isotope measurements were made for each steroid; i.e., 13 duplicate analyses. A few samples were lost or contaminated, and occasionally only single measurements were made on a sample. As a representative analysis, Table 1 gives all of the measurements for Subject 2. Using these data, there are two features of accuracy and reproducibility that can be quantified. One is the variation in δ measurement between duplicate analyses of the same sample and the other the overall variation occurring during analyses of a complete series from one individual. For duplicates, the reproducibility was excellent and did not differ significantly between the five subjects studied. Considering the data shown in Table 1, averages and standard deviations were measured for each duplicate for the two androstanediol and pregnanediol diacetates that give values in the $\delta^{13}C^0/00$ −23 to −30 range. Means were then determined of the standard deviations. For pregnanediol, the mean standard deviation between duplicates was ± 0.21 with a range of ± 0.02 to ± 0.71. Comparable results were obtained for the androstanediols (± 0.11 for 5α-androstanediol and ±0.25 for 5β-androstanediol). These results were considered to represent excellent reproducibility and (as previously noted) suggested that the methodology could be used with *single* determinations by GC/C/IRMS of $\delta^{13}C^0/00$ for each steroid. This was illustrated graphically in Figure 4B and C, which showed both the $\delta^{13}C^0/00$ values for the average of duplicates and for the first analysis. The graphs were essentially identical.

Within a 17-day series of datapoints for duplicate assays for a single individual, the results show considerably less reproducibility—notice the peaks and valleys of the pregnanediol plots in Figure 4. In any physicochemical analyses, it is, of course, possible to have excellent reproducibility but compromised values. For accuracy and reproducibility assessment between a series of different samples, we use the δ measurement of the ERC pregnanediol. The administration of testosterone should in no way change the $\delta^{13}C^0/00$ value for pregnanediol that has to be produced from cholesterol via several intermediates. In a perfect analysis, the measured ^{13}C content of pregnanediol would not be expected to change during the 17-day study period unless, of course, the individual alters his or her diet markedly during the study period. Even with dietary modification, the ^{13}C content of body molecular constituents would be expected to change only slowly.

The highest and lowest duplicate average $\delta^{13}C^0/00$ values for pregnanediol for the five subjects differed by −0.92 to −2.96. As previously mentioned, with the exception of Subject 2, the average duplicate values seemed to rise and fall randomly during the 17-day study period. What

Table 1 $\delta^{13}C^0/_{00}$ values obtained from duplicate GC/C/IRMS analyses for Subject 2 before and following testosterone administration

	Subject 2		
	5βAD	5αAD	PD
Day 1	−26.55	−27.33	−24.93
	−26.15	−27.20	−25.34
	−26.66	−27.28	−25.15
Day 2	−25.68	−27.42	−25.20
	−25.95	−27.49	−25.17
Day 3[a]	−29.33	−29.36	−25.88
	−28.63	−28.81	−25.61
Day 4	−28.99	−29.19	−25.06
	−28.88	−29.24	−25.21
Day 5	−29.34	−29.49	−24.78
	−29.48	−29.61	−24.87
Day 6	−29.37	−29.11	−25.26
	−29.12	−29.16	−24.97
Day 7	−29.33	−29.42	−25.22
	−29.31	−29.28	−25.46
Day 8	−29.16	−29.16	−25.09
	−29.18	−29.14	−25.30
Day 9	−30.06	−28.98	−25.88
	−28.83	−28.78	−25.52
Day 11	−27.41	−28.78	−25.99
	−27.63	−28.88	−24.99
Day 13	−27.47	−27.42	−25.05
	−27.12	−27.59	−25.72
Day 15	−26.39	−26.50	−24.84
	−25.50	−26.28	−24.96
Day 17	−25.24	−26.12	−24.86
	−25.36	−26.32	−25.13

Abbreviations: 5βAD, 5β-androstane-3α,17β-diol; 5αAD, 5α-androstane-3α, 17β-diol and PD, pregnanediol
[a]The first collection following testosterone administration.

USADA 1246

is the source of this irreproducibility? Because we know that the reproducibility of $\delta^{13}C^0/00$ measurements of individual steroids in duplicate GC/C/IRMS runs is excellent, we must assume that the *measured* $\delta^{13}C^0/00$ values for individual chromatographic peaks are accurate. If these are accurate, then any difference in the value from the "true" value for the steroid determined must represent some minor contamination of the steroid peak by components with greater or less $\delta^{13}C^0/00$ values. We have proposed that the trend to increasing values for Subject 2 may be because of dietary changes toward foodstuffs with greater ^{13}C content.

These minor irreproducibilities, which typically give rise to a range of about −1.5 in the δ value for all datapoints during the study period, will not affect the outcome of a drug test, because in no cases do the lowest values breach the discriminant value for the positives.

Testosterone/epitestosterone ratios

Table 2 gives the T/E values for five individuals studied here (Subjects 1–5) and three other subjects whose steroids were not analyzed by the IRMS technique (Subjects 6–8). The data show that the maximal increase in the ratio for each of the individuals was between 12 and 68 times baseline level, although it was noticeable that only 4 of the 8 subjects gave T/E values greater than 6 and 1 of these only modestly exceeded it on one occasion (T/E 6.69). The T/E ratio of the five subjects studied here have been included in Figure 5C as compared to the $\delta^{13}C^0/00$ AD/PD ratio for the same individuals.

$\delta^{13}C^0/00$ steroid values for different nationalities

Figure 6 shows the $\delta^{13}C^0/00$ values for duplicate analyses of the androstanediols and PD from 15 individuals of 11 different nationalities in addition to the Chinese. In some cases, it was possible only to obtain values for the 5β androstanediols because of the low amounts of 5αAD present. The results demonstrate that there are differences among racially and regionally varied individuals with undetermined diet, but we must remember that part of this variation may be attributable to the analytical inaccuracies previously discussed. However, all values were greater than −28.2, which should be compared to the values of less than −29.2

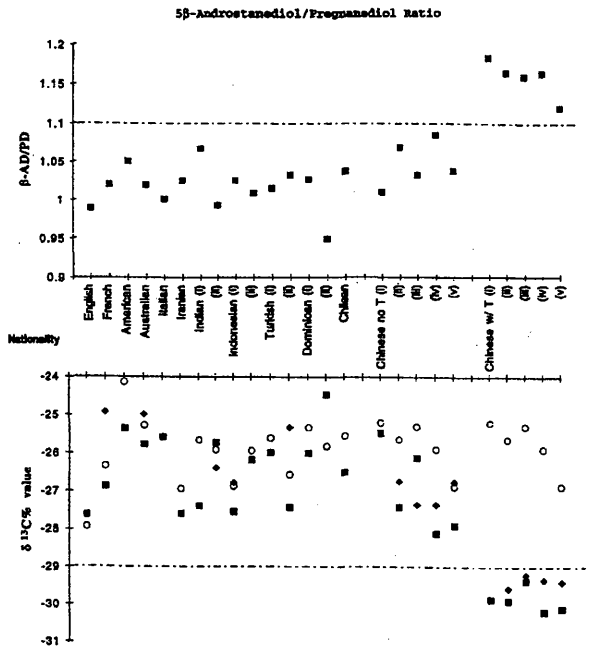


Figure 6 The lower panel shows the $\delta^{13}C^0/00$ values for the androstanediols and pregnanediols for individuals of different nationality, while the upper panel shows the 5βAD/PD ratio. The open circles represent PD, the closed diamonds 5αAD and closed rectangles 5βAD. The baseline values for the Chinese represent the mean of the two measurements, while the Chinese "testosterone" values represent the average of the 7 days after drug administration. Pregnanediol was below detection level in the Chilean urine, so pregnanetriol values were reported instead.

found in the subjects studied following testosterone administration. The mean value for the 5βAD/PD ratio was 1.02 ± 0.03 with a range of 0.95 to 1.07, which compares to the average value for the Chinese baseline samples of 1.05 ± 0.03 (range 1.01 to 1.08). The ratio values for the testosterone administered subjects were all >1.15 in the first days after injection. These data support the previous cut-off ratio value of 1.1:1.0 suggested on the basis of studying the Chinese subjects before and after testosterone administration.

There are notable features with regard to the ^{13}C content

Table 2 Urinary testosterone/epitestosterone ratios for the eight subjects

Study day	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subjec 7	Subject 8
1	0.26	1.28	4.69	0.10	0.12	0.17	0.08	0.11
2	0.33	1.48	5.26	0.11	0.11	0.22	0.06	0.11
3[a]	0.65	8.77	29.3	0.26	0.7	1	1.07	0.42
4	1.95	24.2	36.9	1.77	6.69	4.13	1.36	2.98
5	2.81	45.8	41.8	2.01	5.91	3.53	1.53	7.23
6	2.79	33.2	61.8	1.96	3.71	3.10	1.45	7.48
7	3.03	15.4	54.9	1.91	4.24	3.65	1.68	7.16
8	4.13	36.4	57.7	1.48	5.95	3.08	0.97	4.89
9	5.3	20.3	39.7	1.51	5.78	1.43	1.04	3.81
11	3.2	26.5	33.0	0.58	3.69	0.97	0.40	3.69
13	0.77	2.63	3.42	0.68	3.41	0.20	0.07	1.93
15	0.22	0.99	4.37	0.19	2.27	0.15	0.06	0.21
17	0.26	2.02	4.80	0.13	0.21	0.20	0.08	0.08

[a]Day of testosterone administration.

USADA 1247

of steroids in urine from individuals of different countries. One is that there is a considerable spread in values but one that is unrelated to race and "typical regional diets," at least based on the small sample studied. The lowest and highest $\delta^{13}C^0/00$ among this group differed by 4 units (−24 to −28); however, both these sets of values came from Caucasians. The remaining samples from different nationalities and ethnic groups fell within this range, a particularly important finding with respect to the current study, because it suggests that the results obtained following testosterone administration are universally applicable.

Diet of individuals must have the greatest part to play in the establishment of an individual's $\delta^{13}C$ value. Because all food originates as plant life (including animal protein), it was interesting to research what is known of $\delta^{13}C^0/00$ values for dietary plants. Smith and Epstein[3] published the ^{13}C content of 104 plants, but relatively few were commonly used foodstuffs. A few were high $^{13}C/^{12}C$ plants (corn, sugar cane, grain, and sorghum) averaging around $\delta^{13}C^0/00$ −14, but most were categorized as a separate group of low $\delta^{13}C^0/00$ species having values between −23 and −30. These include dietary oil precursors (olive, sunflower, and castor) and various vegetables (wheat, grass, bamboo, peas, squash, radish, beets, and citrus fruit). Because typical worldwide diets of human and domestic animals use mostly low $\delta^{13}C^0/00$ plants, we would anticipate that $\delta^{13}C$ values for excreted steroids would fall in the −23 to −28 range, as found. The $\delta^{13}C^0/00$ value of an individual plant species does not vary significantly dependent on geographic location,[3] and variation only manifests itself in plants grown in urban areas where CO_2 from fossil fuels increases the ^{12}C content slightly. This clearly does not markedly affect agricultural foodstuffs. For the purposes of this assay technique, it is fortuitous that synthetic testosterone is obviously derived from a very low $\delta^{13}C^0/00$ plant species, otherwise no distinction would be possible between endogenous and exogenous compound.

Practicality of methodology for use in doping control

We compared our $\delta^{13}C^0/00$ values for androstanediol with those of Aguilera et al., who used similar methodologies but different instrumentation.[5] They studied individuals of different nationality and ethnicity than ours. For the purposes of this comparison, we averaged our values for 5α- and 5β-androstanediol, because their values were based on measurement of the combination. For baseline samples, we obtained an averaged $\delta^{13}C^0/00$ of −26.87, which agreed excellently with Aguilera's[5] value of −26.52. For androstanediols measured during testosterone administration, they obtained an average value of −32.44; whereas, our value (the mean of duplicates of the lowest values for each individual) was −30.21. Although our minimum value is not as low as that of Aguilera, it was still well below the baseline level, so it could be used for proving testosterone abuse. The difference in results between the laboratories could be attributed to their use of a testosterone with lower $\delta^{13}C^0/00$ (its origin was not reported) or unequal calibration of the different instruments used.

Our methodology is multistep, albeit built around low-technology procedures. Within the technique, there is only one chromatographic separation, and that was designed to produce a broad fraction rather than the isolation of individual steroids. The key to the method is separate isolation of nonketonic steroids, a procedure that in one extraction step removes 75% of unwanted steroids from urine. A second classical procedure, the oxidative removal of side chains from 17-hydroxypregnane steroids removes many of the remaining complex steroids from the mixture, resulting in a cleaner chromatogram, although in the future, we may simplify the method by discontinuing this procedure if satisfactory results can be obtained. Typically, without using any automation in wet chemistry procedures, a batch of samples requires 1 day of technician time to extract and derivatize. The mass spectrometry was automated, and latterly 20 minutes were required for each run. The maximum number of samples that could be analyzed per 24 h was, therefore, 72.

The length of time that the 250-mg testosterone enanthate dose (equivalent to 180-mg free testosterone) clearly manifested itself in decreased $\delta^{13}C^0/00$ value was 8–10 days. This compares well with other parameters studied on this cohort. For example, plasma testosterone was measured, and from being grossly elevated in the first few days after administration, it returned to normal by the 8th day, although the steroid itself may well be largely of exogenous origin on this occasion.[11] In addition, we report the T/E ratios that are particularly interesting, because essentially only three out of the eight individuals achieve values >6 on more than 1 day, showing a high rate of false negatives. This may not be a universal finding and may well be related to the race (Asian) of the studied individuals. However, it once again emphasizes the importance of developing alternate methodology for proving testosterone misuse. The elevated T/E ratios (although most are <6) fall significantly by the 11th day of the study in all subjects.

We believe that our method based on androstanediol measurement has the needed sensitivity for use in doping control. Although we analyzed all samples in duplicate and plotted the graphs from their averages, individual analyses were equally informative (Figure 4B, C). We would have no hesitation in confirming testosterone administration based on a single measurement. We have been able to get up to five analyses of a single sample using splitless gas chromatographic injection. This represents an equivalent of 5 mL urine for each analysis, which meets sample availability in doping control. Use of a solid injection device for GC, such as glass minivials in a carousel,[12] could improve this sensitivity greatly, because the total sample could be volatized and passed into the GC/IRMS instrument. Testing of a urine volume of 2 mL by our procedure and the Micromass IRMS instrument would be realistic. Our method has two obvious advantages over that of Aguilera et al.[5] One is that the two androstanediols are determined separately, so even if one peak was too small or showed evidence of contamination, the second peak could give the required value. A second advantage is that all analytes are measured in the same chromatogram; whereas, in Aguilera et al.'s study, at least two separate GC/C/IRMS analyses are required to measure the isotope ratio in the androstanediols and their

USADA 1248

ERC cholesterol acetate. However, in fairness, they did have a more difficult assay through the inclusion of testosterone itself among the analytes.

Manipulating the result of a drug test to give a "negative" result following testosterone administration would be difficult. Although the AD/PD ratio could be normalized by taking commercial pregnanediol (Sigma, $\delta^{13}C^0/00$ value −32.12) the low "absolute" value for pregnanediol would give the situation away. Similarly, commercial androstanediols also have very low $\delta^{13}C^0/00$ values (Sigma −34.00) so self-administration of these compounds would not increase the δ values of testosterone derived metabolites. Probably the only way to fool such a test would be to alter the diet drastically over a long period of time through exclusively ingesting foodstuffs with $\delta^{13}C^0/00$ values below −29. False positives could be obtained by ingestion of nonproscribed steroids such as the now commonly used dehydroepiandrosterone (DHEA), which also partially metabolizes to androstanediols. However, whether testosterone or DHEA was being administered could easily be determined from marked differences in the total urinary steroid profile.

Acknowledgments

These studies were made possible through the generous support of the U.S. Olympic Committee and its representatives Dr. Wade Exum and Dr. James Betts. Financial support was also provided by Children's Hospital Oakland Research Institute. The authors thank Dr. Victor Rogozkin (St. Petersburg), Dr. Lubos Stárka (Prague), and Drs. Xavier de la Torre and Jordi Segura (Barcelona) for donating synthetic testosterone standards. Thanks are also given to individuals from different countries who provided urine samples for analysis. These studies could not have been carried out without the continual mass spectrometric maintenance and training undertaken by Ms Esther Roitman.

References

1. Catlin DH, Cowan DA, De la Torre R, Donike M, Fraisse D, Oftegro H, Hatton CK, Starcevic B, Becchi M, de la Torre X, Norli H, Geyer H, Walker CJ (1996). Urinary testosterone (T) to epitestosterone (E) ratios by GC/MS. I. Initial comparison of uncorrected T/E in six international laboratories. *J Mass Spectrom* **31**:397–402.
2. Southan G, Mallet A, Jumeau J, Craig S, Poojara N, Mitchell D, Wheeler M, Brooks RV (1990). Programme and Abstracts of The 2nd International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona, Spain, p. 306.
3. Smith BN, Epstein S (1971). Two categories of $^{13}C/^{12}C$ ratios for higher plants. *Plant Physiol* **47**:380–384.
4. Becchi M, Aguilera R, Farizon Y, Flament IM-M, Casabianca H, James P (1994). Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rapid Commun Mass Spectrom* **8**:301–308.
5. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope JHG (1996). Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom* **31**:169–176.
6. Donike M, Bärwald KR, Klosterman K, Schänzer W, Zimmerman J (1983). *Sport.* Leistung und Gesundheit, Cologne, Germany, p. 293.
7. Shackleton CHL, Whitney JO (1980). Use of Sep-pak® cartridges for urinary steroids extraction: Evaluation of the method for use prior to gas chromatographic analysis. *Clin Chim Acta* **107**:231–243.
8. Girard A, Sandulesco G (1936). Sur une nouvelle série de réactif du groupe carbonyle, leur utilisation à l'extraction des substances cétoniques et à la characterisation microchinique des aldehydes et cétones. *Helv Chim Acta* **19**:1095–1107.
9. Bush IE (1961). *The Chromatography of Steroids.* Pergamon Press Ltd., Oxford, UK, pp. 358–371.
10. Setchell KDR, Shackleton CHL (1973). The group separation of plasma and urinary steroids by column chromatography on Sephadex LH-20. *Clin Chim Acta* **47**:381–388.
11. de la Torre X, Segura J, Farré M, Yang Z, Li Y, Wu M (1995). Steroid profile in different ethnic groups after testosterone ethanate administration. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke U (eds), *Recent Advances in Doping Analysis.* Sport und Buch Strauß, Köln, Germany, pp. 59–80.
12. Shackleton CHL, Honour JW (1976). Simultaneous estimation of urinary steroids by semiautomated gas chromatography. Investigation of neonatal infants and children with abnormal steroid synthesis. *Clin Chim Acta* **69**:267–283.

USADA 1249

TAB 41

UNION CYCLISTE INTERNATIONALE
CH 1860 Aigle / Suisse
Tél. : +41 24 468 58 11 - Fax : +41 24 468 58 68 - e-mail : christian.varin@uci.ch

FAX MESSAGE

To	:	USA CYCLING Mr. Steve JOHNSON
Fax nbr.	:	+1 719 866 46 28
From	:	Christian Varin, Manager
Copy		AGENCE MONDIALE ANTIDOPAGE Mme Janie Soublière +1 514 904 45 45 USADA Mr. Travis Tygart +1 719 785 20 01 PHONAK HEARING SYSTEMS Mr. John Lelangue 055-254.70.11
Date	:	July 26th 2006
Ref	:	Anti-Doping Services/ Cv/Dl
Total pages	:	6 (including this one)
Subject	:	*File 29/06*

URGENT
CONFIDENTIEL

This facsimile may contain information that is confidential and which may be subject to legal privilege. If you are not the intended recipient, you must not pursue, use, disseminate or copy this message. If you received this message in error, please notify us by telephone (+41 24.468.58.11) and return the original message by mail. Thank you.

AVERTISSEMENT : Le contenu du présent fax ainsi que les documents qui y sont joints sont protégés par le secret professionnel. Toute communication, copie ou révélation de leur contenu à une personne autre que leur destinataire est strictement interdite et pénalement sanctionnée. Au cas où ce fax ne vous serait pas destiné nous vous remercions de bien vouloir nous en aviser immédiatement par téléphone (+41.24.468.58.11) et nous retourner l'original par courrier.

INTERNATIONAL CYCLING UNION

Registered / Confidential
USA CYCLING
Mr. Steve JOHNSON
One Olympic Plaza
CO-80909 COLORADO SPRINGS
USA

Aigle, July 26th 2006
Ref: Antidoping / Cv / DI

File Nr. 29/06 (to mention in your correspondence)

Floyd LANDIS (licence 0020272 - UCI Code USA19751014) / Tour de France (FRA), 20th July 2006

Dear Sir,

We inform you that the rider Floyd LANDIS tested positive (T/E) at the above-mentioned race according to the report we received from the Laboratory of Châtenay-Malabry (FRA), a copy of which we enclose (pursuant to article 187 of the Anti-Doping Rules). According to the laboratory's result on the A sample, the Anti-doping Commission must start from this notion that an offence against the mentioned Regulations has objectively been committed.

The rider is allowed to require without delay the B sample analysis (in accordance with articles 191 and following of the Anti-Doping Rules), or, failing that, it will be considered that he has renounced to this right. Mr. Floyd LANDIS and/or his representative have also the right to attend the opening of the B sample and his analysis if this one is required. The rider is also allowed to require a copy of the complete analysis report for the samples A and B. In accordance with article 194 AR, to be acceptable the request for the analysis of the B sample shall be sent by the national federation no more than 5 working days after receipt of the letter informing it of the adverse analytical finding.

Please be reminded that, according to the Anti-doping Regulations - chapter X, the final sanction is foreseen by art. 261 and reads as follows:

Art. 261

Imposition of Ineligibility for Prohibited Substances and Prohibited Methods

Except for the specified substances identified in article 262, the period of Ineligibility imposed for a violation of article 15.1 (presence of Prohibited Substance or its Metabolites or Markers), article 15.2 (Use or Attempted Use of Prohibited Substance or Prohibited Method) and article 15.6 (Possession of Prohibited Substances and Methods) shall be:

First violation: *2 (two) years' Ineligibility*

Second violation: *lifetime ineligibility*

However, the License-Holder shall have the opportunity in each case, before a period of Ineligibility is imposed, to establish the basis for eliminating or reducing this sanction as provided in articles 264 and 265.

We would like to remind you that a violation of these Anti-Doping Rules in connection with an In-Competition test automatically leads to Disqualification of the individual result obtained in that Competition according to article 256. In addition, we also ask you to take into consideration the articles 257 to 260.

Regarding the cost of the proceedings, please refer to articles 244 to 246.

Moreover, we communicate you the text of the article 9.2.002 of the UCI Regulations which provides:

A rider against whom an investigation was opened in relation to a fact which may cause a breach of the UCI Anti-Doping Rules, will not be eligible for the World Championships until the end of the suspension or until his definitive acquittal. In the event of a positive A sample, this clause applies starting from the notification of the abnormal analysis result to the rider.

Unless otherwise decided by the anti-doping commission, the above paragraph is also applicable in the event of an investigation or a procedure regarding such a fact, opened in pursuance of a law or other regulation.

Specific cases are examined by the anti-doping commission or its president. Their decision is without appeal.

In addition to the disqualification, the licensee and his national federation will be respectively sanctioned by a fine of CHF 2000 to CHF 10000.

The present condition for participation, aims to protect the integrity, serenity and reputation of the World Championships. Its application does not prejudge the decision whether an anti-doping violation has occurred and shall not give rise to any claim in the events of acquittal.

We would consequently ask you to implement proceedings according to articles 188 to 205 of those Regulations. We would remind you that, according to the articles 249 to 251, you have to keep us informed of all measures you take by sending us copies thereof.

Looking forward to hearing from you.

Sincerely yours,

Anti-doping Services,

Christian VARIN, Manager

Enc.: mentioned

Copy to: WADA
USADA
PHONAK HEARING SYSTEMS

PROCES-VERBAL DE CONTROLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP ROAD CA 92562 MURRIETA (USA)

N° de licence : 0020272

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14 10 1975

Sexe / Sex : M ☒ F ☐

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Fédération – Discipline / Sport : UCI – FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 17ème ETAPE

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 20/07/2006

Heure de remise de la notification : 17 h 10

Lieu du contrôle / Place of test : MORZINE AVORIAZ UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : LANDIS
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : MERAVIGLIA Giovanni

Nom et prénom du médecin / Name of doctor : (en toutes lettres) BORDAGERRY GERARD

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival :	Date du prélèvement / Sampling date :	Heure du prélèvement / Sampling time :
17 h 50	20/07/2006	17 h 55

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 995474

Quantité totale d'urine, sang, ml
Total amount of urine, blood, ml : 125 ml

Densité / Specific Gravity : 1.030

pH / pH : 5

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :	Refus / Refusal	Oui	Non	Empêchement / Obstacle	Oui	Non

Médicaments / Drugs ☒ OUI ☐ NON

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

05.05.06 Vaccination Tetanol Virale (Aout)
05.05.06 Vaccination Tetanol Virale (Aout)
20.07.06 Prise de Xylocaine injectable (Aout)
20.07.06 Prise de Xylocaine injectable (Aout)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : Une Bouteille Urinaire Percée

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate :

Signature du sportif contrôlé : Signature of person tested :	Signature du Médecin : Signature of Doctor :	Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

0092221

VERSION NATIONALE

LNDD
LABORATOIRE NATIONAL DE DÉPISTAGE DU DOPAGE

cofrac
ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 25 juillet 2006

28423

RAPPORT D'ANALYSE N° 178/07-1

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BORDABERRY

Sport : **Cyclisme (UCI)**
Épreuve et lieu : **TDF 2006 : 17ème étape à Morzine**
Date : **20/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date : **20/07/2006**
Type de matériel : **Berlinger**
Nombre d'échantillon(s) : **1 (sur 3 échantillons reçus)**
Référence de(s) l'échantillon(s) : **178/07_995474**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale ; Contrôle en compétition)

Date de début des analyses : **21/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C*, ES04, ES05*) et liquide (ES03, ES03B*) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B*).

Conclusions : *(pH = 5,2 d = 1.025 +/- 0.002)*

T/E estimé à 11.4 *(variation maximale admissible = 30%)*

Concentration de Testostérone estimée après correction par la densité à 45.4 ng/mL
(Variation maximale admissible = 20 %)

Concentration d'Epitestostérone estimée après correction par la densité à 3.9 ng/mL
(Variation maximale admissible = 30 %)

Rapport supérieur au seuil de 4

* *Hors portée de l'accréditation COFRAC.*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 2 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : +33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

RAPPORT D'ANALYSE N° 178/07-1 (SUITE)

Conclusions (Suite) :

L'analyse complémentaire par spectrométrie de masse de rapport isotopique indique une origine exogène des métabolites de la Testostérone, cohérente avec une prise de Testostérone ou de l'un de ses précurseurs.

L'origine exogène des métabolites de la Testostérone a été objectivée sur la base d'un appauvrissement isotopique de 3.99 ‰ et 6.14 ‰, respectivement pour les métabolites Androstérone et 5α-Androstanediol.

Résultat exprimé par rapport au seuil de positivité de l'AMA :

- Appauvrissement isotopique > à 3 ‰ *(variation maximale admissible appliquée au laboratoire = 0.8‰)*

Dépistage : Chromatographie gazeuse couplée à la spectrométrie de masse (ES04)
Confirmation : Chromatographie gazeuse couplée à la spectrométrie de masse (EC24D) et spectrométrie de masse de rapport isotopique GC-IRMS (EC31)

NB : Autres essais (ES02, ES02C*, ES03, ES03B*, ES05*, ES06 et ES08B*) : Recherche de substances dopantes négative

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale – C. VARIN (CH 1860 AIGLE - SUISSE)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique– 75700 PARIS)

Pour information :
- J. SOUBLIERE : WADA (fax n°00.1.514.904.18.00)
- Dr. GENSON - Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)

* *Hors portée de l'accréditation COFRAC.*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 2 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction(

Linda Barnes

From: Travis Tygart
Sent: Thursday, July 27, 2006 7:56 AM
To: Linda Barnes
Subject: FW: UCI Case 29/06

For the file.

Thanks,

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel United States Anti-Doping Agency 1330 Quail Lake Loop, Suite 260 Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

-----Original Message-----
From: Petty, Sean [mailto:spetty@usacycling.org]
Sent: Wednesday, July 26, 2006 5:50 PM
To: Travis Tygart
Cc: Johnson, Steve
Subject: UCI Case 29/06

Travis,
USA Cycling requests that USADA manage the process for adjudication of UCI case 29/06. The associated documents USA Cycling received from the UCI on this matter were faxed to you today.

Please let me know if you have any questions. Steve and I will be in Brazil tomorrow through Monday but will be available on our cell phones and via e-mail.

Best regards,

Sean Petty
Chief of Staff
USA Cycling
1 Olympic Plaza
Colorado Springs, CO 80909
Phone: 719-866-4783
FAX: 719-866-4596
spetty@usacycling.org
UCI Road Commission Member

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

VIA FACSIMILE

July 27, 2006

Mr. Steve Johnson
Mr. Steve Petty
USA Cycling
One Olympic Plaza
Colorado springs, CO 80909

RE: UCI File No. 29/06
Tour de France, July 20, 2006
Floyd Landis – Sample #995474

Dear Mr. Johnson and Mr. Petty:

I am in receipt of your letter and email to me of yesterday requesting that the United States Anti-Doping Agency ("USADA") handle the adjudication of the above referenced case and forwarding us the documents concerning the case that you have received from the Union Cyclist Internationale ("UCI").

I am writing to inform you that USADA accepts the handling of this matter and will proceed with the management of this case. We will notify the athlete, the UCI and the United States Olympic Committee ("USOC") that we will be handling the matter on your behalf as required by the rules and will copy you on all relevant communications.

Please forward us any documents that you receive from the athlete, the UCI or others concerning this case.

If you have any questions or concerns about this matter, please do not hesitate to contact me.

Sincerely,

Travis T. Tygart
General Counsel

Cc: Christian Varin, Anti-Doping Services, UCI
Gary Johansen, USOC Deputy General Counsel
Jim Scherr, USOC Chief Executive Officer

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260, Colorado Springs, CO 80906 ■ Tel: 719.785.2000 ■ Fax: 719
usada@usantidoping.org ■ www.usantidoping.org

Linda Barnes

From: Travis Tygart
Sent: Friday, July 28, 2006 9:06 AM
To: Varin Christian - UCI
Cc: Linda Barnes
Subject: B Sample.

Importance: High

Christian-

I just left a message for you at the office and with a lovely person who answered your cell phone. Please give me a call concerning a few urgent matters concerning the B sample analysis of your file 29/06 (the Tour de France for an elevated T/E and CIR positive).

You can reach me at the office or on my cell phone (719-785-2031). My office number is below.

Thanks,

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel United States Anti-Doping Agency 1330 Quail Lake Loop, Suite 260 Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028

Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

Johncie Wingard

om: Johnson, Steve [sjohnson@usacycling.org]
Sent: Monday, July 31, 2006 1:22 PM
To: Varin Christian - UCI
Cc: Travis Tygart; lon.schatten@wxs.nl
Subject: RE: Floyd Landis - B sample analysis

Christian,

I am in receipt today of a fax from Floyd Landis to USA Cycling with copies to USADA and UCI requesting the B sample analysis be undertaken.

I am also in receipt today of a fax from Mr. Pat McQuaid indicating that the UCI has interceded in accordance with article 191 of the anti-doping regulations and requested the B sample analysis proceed prior to the closing of the Chatenay-Malabry laboratory for the annual vacation period. Because of this development, I am assuming that no further action is required by USA Cycling to ensure that the B sample analysis will proceed as requested.

As always, thank you for your communication in this important matter. Please do not hesitate to contact me if I you require further action on our part.

All the best,

Steve Johnson

Johnson, CEO
USA Cycling
1 Olympic Plaza
Colorado Springs, CO 80909
719-866-4727
sjohnson@usacycling.org
www.usacycling.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Varin Christian - UCI [mailto:christian.varin@uci.ch]
Sent: Monday, July 31, 2006 2:26 AM
To: Johnson, Steve
Cc: Travis Tygart; lon.schatten@wxs.nl
Subject: Floyd Landis - B sample analysis
Importance: High

Dear Steve,

Th UCI antidoping Commission asked me to send you the following comments:

We refer to articles 184 to 205 of the UCI antidoping Rules. According to article 193, the request for the analysis of the B sample

shall be made directly to the laboratory by the Rider's National Federation, either on its own initiative or at the request of the Rider A copy of the request for the analysis must be sent to the UCI at the same time.

se find here under the laboratory's details:

Laboratoire National de Dépistage du Dopage
CREPS
143, avenue Roger-Salengro
F – 92290 Châtenay-Malabry
France

Tel: + 33 1 46 60 28 69
Fax: + 33 1 46 60 30 17

Email: direction@lndd.com

We assure you that we are at your entire disposal should you need any help from the UCI!

As always, we thank you for your precious collaboration and courtesies in this matter!

Yours sincerely,

Christian Varin
Manager Antidopage / Antidoping Manager
Union Cycliste Internationale
CH - 1860 Aigle

Tél : +41-24.468.58.11
Fax : +41-24.468.58.12
v uci.ch

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

VIA FACSIMILE

July 31, 2006

Christian Varin, Manager
Anti-Doping Services
Union Cycliste International
Ch 1860 Aigle
Switzerland

J. de Ceaurriz, Director
Laboratoire National de Depistage du Dopage
143, avenue Roger Salengro
92290 Chatenay-Malabry
FRANCE

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

te Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

RE: UCI File No. 29/06
Tour de France, July 20, 2006
Floyd Landis – Sample #995474

Gentlemen:

I am in receipt of the letter from UCI dated July 31, 2006, and the letter of today from the Mr. Landis requesting the B sample opening and analysis.

I am writing to confirm that the B sample opening and analysis of sample #995474 will begin on Thursday, August 3, and conclude on Saturday, August 5, 2006. USADA understands that that the B sample confirmation will include the testosterone/epitestosterone analysis and the carbon isotope ratio analysis.

Additionally, the athlete will be represented by Mr. José M. Buxeda and Dr. Douwe de Boer. USADA will also have a representative, Ms. Caroline Hatton, attend the B sample opening and analysis.

If you have any questions or concerns about this matter, please do not hesitate to contact me.

Sincerely,

Travis T. Tygart
General Counsel

cc: Sean Petty, USA Cycling (w/o encls.)
Gary Johansen, USOC Deputy General Counsel
Jim Scherr, USOC Chief Executive Officer
Mr. José M. Buxeda & Mr. Luis Sanz Hernández, Counsel for Mr. Landis

Linda Barnes

From: Travis Tygart
Sent: Monday, July 31, 2006 7:54 AM
To: Varin Christian - UCI; Johnson, Steve
Cc: lon.schatten@wxs.nl; Linda Barnes
Subject: RE: Floyd Landis - B sample analysis

Steve-

We have informed the rider to send his request to USADA sine we have accepted the handling of the matter from you. In the event you receive the request for the B sample, please forward it to USADA. Likewise, we will forward you any correspondence that we receive concerning the B sample request.

Thanks,

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Varin Christian - UCI [mailto:christian.varin@uci.ch]
Sent: Monday, July 31, 2006 2:26 AM
To: Johnson, Steve
Cc: Travis Tygart; lon.schatten@wxs.nl
Subject: Floyd Landis - B sample analysis
Importance: High

Dear Steve,

The UCI antidoping Commission asked me to send you the following comments:

We refer to articles 184 to 205 of the UCI antidoping Rules. According to article 193, the request for the analysis of the B sample shall be made directly to the laboratory by the Rider's National Federation, either on its own initiative or at the request of the Rider. A copy of the request for the analysis must be sent to the UCI at the same time.

Please find here under the laboratory's details:

Laboratoire National de Dépistage du Dopage
CREPS
143, avenue Roger-Salengro

7/31/2006

F – 92290 Châtenay-Malabry
France

Tel: + 33 1 46 60 28 69
Fax: + 33 1 46 60 30 17

Email: direction@lndd.com

We assure you that we are at your entire disposal should you need any help from the UCI!

As always, we thank you for your precious collaboration and courtesies in this matter!

Yours sincerely,

Christian Varin
Manager Antidopage / Antidoping Manager
Union Cycliste Internationale
CH - 1860 Aigle

Tél : +41-24.468.58.11
Fax : +41-24.468.58.12
www.uci.ch

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

VIA FACSIMILE

July 31, 2006

Christian Varin, Manager
Anti-Doping Services
Union Cycliste International
Ch 1860 Aigle
Switzerland

J. de Ceaurriz, Director
Laboratoire National de Depistage du Dopage
143, avenue Roger Salengro
92290 Chatenay-Malabry
FRANCE

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

.ate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

RE: UCI File No. 29/06
Tour de France, July 20, 2006
Floyd Landis – Sample #995474

Gentlemen:

I am in receipt of the letter from UCI dated July 31, 2006, and the letter of today from the Mr. Landis requesting the B sample opening and analysis.

I am writing to confirm that the B sample opening and analysis of sample #995474 will begin on Thursday, August 3, and conclude on Saturday, August 5, 2006. USADA understands that that the B sample confirmation will include the testosterone/epitestosterone analysis and the carbon isotope ratio analysis.

Additionally, the athlete will be represented by Mr. José M. Buxeda and Dr. Douwe de Boer. USADA will also have a representative, Ms. Caroline Hatton, attend the B sample opening and analysis.

If you have any questions or concerns about this matter, please do not hesitate to contact me.

Sincerely,

Travis T. Tygart
General Counsel

cc: Sean Petty, USA Cycling (w/o encls.)
Gary Johansen, USOC Deputy General Counsel
Jim Scherr, USOC Chief Executive Officer
Mr. José M. Buxeda & Mr. Luis Sanz Hernández, Counsel for Mr. Landis

Johncie Wingard

From: Varin Christian - UCI [christian.varin@uci.ch]
Sent: Tuesday, August 01, 2006 1:55 AM
To: Johnson, Steve
Cc: Travis Tygart; lon.schatten@wxs.nl
Subject: RE : Floyd Landis - B sample analysis

Dear Steve,

UCI offices are closed today and I am out of office. I will confirm everything directly with USADA as from tomorrow.

The laboratory should confirm that the counter analysis will start at 9 am on thursday 3rd August 2006.

We have taken due note that Mr. Landis and USADA have designated experts.

The UCI will also designate an expert: Mr. Martial Saugy

Yours sincerely,

Christian Varin

-------- Message d'origine--------
De: Johnson, Steve [mailto:sjohnson@usacycling.org]
Date: lun. 31/07/2006 21:21
Ã: Varin Christian - UCI
Cc: Travis Tygart; lon.schatten@wxs.nl
Objet : RE: Floyd Landis - B sample analysis

Christian,

I am in receipt today of a fax from Floyd Landis to USA Cycling with copies to USADA and UCI requesting the B sample analysis be undertaken.

I am also in receipt today of a fax from Mr. Pat McQuaid indicating that the UCI has interceded in accordance with article 191 of the anti-doping regulations and requested the B sample analysis proceed prior to the closing of the Chatenay-Malabry laboratory for the annual vacation period. Because of this development, I am assuming that no further action is required by USA Cycling to ensure that the B sample analysis will proceed as requested.

As always, thank you for your communication in this important matter. Please do not hesitate to contact me if I you require further action on our part.

All the best,

Steve Johnson

Steve Johnson, CEO
USA Cycling
1 Olympic Plaza

Colorado Springs, CO 80909
719-866-4727
sjohnson@usacycling.org <mailto:sjohnson@usacycling.org> www.usacycling.org
<http://www.usacycling.org>

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Varin Christian - UCI [mailto:christian.varin@uci.ch]
Sent: Monday, July 31, 2006 2:26 AM
To: Johnson, Steve
Cc: Travis Tygart; lon.schatten@wxs.nl
Subject: Floyd Landis - B sample analysis
Importance: High

Dear Steve,

The UCI antidoping Commission asked me to send you the following comments:

e refer to articles 184 to 205 of the UCI antidoping Rules. According to article 193, the request for the analysis of the B sample shall be made directly to the laboratory by the Rider's National Federation, either on its own initiative or at the request of the Rider. A copy of the request for the analysis must be sent to the UCI at the same time.

Please find here under the laboratory's details:

Laboratoire National de Dépistage du Dopage

CREPS

143, avenue Roger-Salengro

F - 92290 Châtenay-Malabry

France

Tel: + 33 1 46 60 28 69

Fax: + 33 1 46 60 30 17

ail: direction@lndd.com

We assure you that we are at your entire disposal should you need any help from the UCI!

As always, we thank you for your precious collaboration and courtesies in this matter!

Yours sincerely,

Christian Varin

Manager Antidopage / Antidoping Manager

Union Cycliste Internationale

CH - 1860 Aigle

Tél : +41-24.468.58.11

Fax : +41-24.468.58.12

www.uci.ch <http://www.uci.ch/>

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

USADA 0388

Johncie Wingard

om: Johnson, Steve [sjohnson@usacycling.org]
Sent: Saturday, August 05, 2006 3:59 PM
To: Travis Tygart
Subject: Floyd Landis

Travis,

I just want to confirm that our previous request for USADA to manage this case includes the new disciplinary phase as well.

Thanks,

Steve

Steve Johnson, CEO

USA Cycling

1 Olympic Plaza

Colorado Springs, CO 80909

7 66-4727

sjohnson@usacycling.org

www.usacycling.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

INTERNATIONAL CYCLING UNION
CH 1860 Aigle / Switzerland
✆ : +41 24 468 58 11 – Fax : +41 24 468 58 12 – e-mail : christian.varin@uci.ch

FAX MESSAGE

To	:	USA CYCLING Mr. Steve JOHNSON
Fax nbr	:	+ 1 719 866 41 02
From	:	Christian VARIN
Cc	:	WORLD ANTIDOPING AGENCY Mrs. Janie SOUBLIERE + 1 514 904 45 45
		USADA Mr. Travis TYGART + 1 719 785 20 01
		PHONAK HEARING SYSTEMS Mr.John LELANGUE 055-254 70 11
		LAW OFFICES OF HOWARD L. JACOBS Mr. Howard JACOBS + 1 818 292 87 36
Date	:	5th August 2006
Ref	:	Antidoping / cv
Total pages	:	4 (including this one)
Subject	:	**File 29/06**

Dear Mr Johnson,

Please find enclosed a copy of Châtenay-Malabry's laboratory report concerning sample "B" n° 995474!

The "B" sample analysis confirms the result of the "A" sample analysis!

T/E ratio is 11. Also, the presence of exogenous testosterone is confirmed!

According to article 224 of the UCI Anti-Doping Rules rules: *" When, following the results management process described in chapter VII, the Anti-Doping Commission makes an assertion that these Anti-Doping Rules have been violated, it shall notify the License-Holder's National Federation and request it to instigate disciplinary proceedings. It shall also send a*

copy of the test analysis report and/or other documentation. A copy of the statement may be sent to the License-Holder and/or the License-Holder's club or team.

A copy of the statement is sent to WADA and to the License-Holder's National Anti-Doping Organization."

Following the "B" sample analysis and the report issued by the laboratory, the UCI Anti-Doping Commission formally requests your Federation / USADA to instigate disciplinary proceedings in accordance with the UCI Anti-Doping Rules!

We are looking forward to hearing from you!

Yours sincerely,

Antidoping Services,

Christian Varin, Manager

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

28541

Châtenay-Malabry, le 05 août 2006

ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

RAPPORT DE CONTRE ANALYSE

Demandes de contre-analyse de l'UCI et de l'USADA du 31 juillet 2006 suite au rapport d'analyse n°178/07-I

Prélèvements du Dr. BORDABERRY

Organisme : Cyclisme (UCI)
Épreuve et lieu : TDF 2006 : 17ème étape à MORZINE
Date : 20/07/2006

Réception de(s) l'échantillon(s) d'urine :
Date : 20/07/2006
Type de matériel : Berlinger
Nombre d'échantillon(s) : 1 (sur 3 échantillons reçus)
Référence de(s) l'échantillon(s) : 178/07_995474 B

Résultats (Cf référentiels en vigueur : AMA / Réglementation nationale – contrôle en Compétition)

Date de début des analyses : 03/08/2006

Méthodes utilisées : Chromatographie gazeuse couplée à la spectrométrie de masse GC/MS (EC24D) et Spectrométrie de masse de rapport isotopique GC/C/IRMS (EC31)

Conclusions : *(pH = 5.2 d = 1.025 +/- 0.002)*

T/E estimé à 11.0 *(variation maximale admissible = 30%)*

Concentration de Testostérone estimée après correction par la densité à 45.7 ng/mL
(Variation maximale admissible = 20 %)

Concentration d'Epitestostérone estimée après correction par la densité à 4.2 ng/mL
(Variation maximale admissible = 30 %)

Rapport supérieur au seuil de 4

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 2 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 e mail : LNDD.FRANCE@wanadoo.fr

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

RAPPORT DE CONTRE ANALYSE (SUITE)

Conclusions (Suite) :

L'analyse complémentaire par spectrométrie de masse de rapport isotopique indique une origine exogène des métabolites de la Testostérone, cohérente avec une prise de Testostérone ou de l'un de ses précurseurs.

L'origine exogène des métabolites de la Testostérone a été objectivée sur la base d'un appauvrissement isotopique de 6.4 ‰ et 3.5 ‰, respectivement pour les métabolites 5α-Androstanediol et Androstérone.

Résultat exprimé par rapport au seuil de positivité de l'AMA :

- Appauvrissement isotopique > à 3 ‰ *(variation maximale admissible appliquée au laboratoire = 0.8‰)*

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale – C. VARIN (CH 1860 AIGLE - SUISSE)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique– 75700 PARIS)

Pour information :
- J. SOUBLIERE : WADA (fax n°00.1.514.904.18.00)
- Dr. GENSON - Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 2 page(s).

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

VIA FACSIMILE

August 7, 2006

Christian Varin, Manager, Anti-Doping Services
Union Cycliste International
Ch 1860 Aigle
Switzerland

RE: UCI File No. 29/06
Tour de France, July 20, 2006
Floyd Landis – Sample #995474

Dear Mr. Varin:

I am in receipt of your letter dated August 5, 2006, forwarding the B sample laboratory results concerning the above referenced case.

As I indicated in my letter of July 31, 2006, the United States Anti-Doping Agency ("USADA") handles the adjudication of potential anti-doping violations in the United States. We have informed the athlete, USA Cycling and the United States Olympic Committee that we will proceed with the above referenced case under the USADA Protocol for Olympic Movement Testing.

At this time, please forward to us as soon as possible the laboratory documentation supporting the finding. I have enclosed, by way of an example, a list of laboratory documents that we typically receive. As you are probably aware, once we receive the laboratory documents, we will forward those to the athlete and notify him that he has ten (10) days to respond in writing. Following that time, an independent anti-doping review board will consider the matter and make a recommendation whether there is sufficient evidence of doping to proceed with the adjudication process. Given this, the sooner we receive the laboratory documents concerning the analysis, the sooner we can begin the ten (10) day time period for the case to be presented to the review board.

Additionally, given that the laboratory results include an elevated testosterone/epitestosterone ratio, please forward to us as soon as possible all prior and any subsequent test results, including the steroid profiles, from this athlete.

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

If you have any questions or concerns, about this matter please do not hesitate to contact me. We look forward to your prompt reply.

Sincerely,

Travis T. Tygart
General Counsel

cc: Sean Petty, USA Cycling
Janie Soubliere, WADA
Gary Johansen, USOC Deputy General Counsel
Jim Scherr, USOC Chief Executive Officer
Mr. Howard Jacobs, Counsel for Mr. Landis

ANNEX D

The following documentation will be supplied as the standard documentation package:

Table of contents
List of laboratory staff involved in the test, including signatures and/or initials and position title(s)
Sample identification information
- Organization requesting the test
- Date of sample collection and site identification
- USADA sample identification number
- Laboratory sample identification number

Urine integrity test results (if completed)
Chain of custody documentation for sample container
- Doping Control Notification form (Laboratory copy)
- Transportation chain of custody (e.g., courier documentation, laboratory receipt of container)
- "A" sample container chain(s) of custody
- Documentation of any deviations from the written screening procedures, if any

"A" Sample Screening Results
- Relevant aliquot chain(s) of custody
- Screening procedure data, including chromatograms (or other relevant data), for Negative control urine
 - Positive control urine (with concentration indicated, if relevant)
 - Sample urine aliquot(s)
 - Analytical run instrument validation data (e.g.; tune data)
- Documentation of any deviations from the written screening procedures, if any

"A" Sample Confirmation Results
- Summary of the analytical principles of the confirmation method
- Aliquot chain of custody
- Sequence verification data
- Confirmation procedure data, including chromatograms (or other relevant data), for
 - Negative control urine
 - Positive control urine (with concentration indicated, if relevant)
 - Standard(s)/calibrator(s) (if relevant)
 - Sample urine aliquot(s)
 - Analytical run instrument validation data (e.g.; tune data)
- "A" sample report (including numerical data for threshold substances*)
 - pH, Specific Gravity, and other urine integrity test results (if applicable, including abnormal appearance of sample) performed in laboratory.
- Documentation of any deviations from the written screening procedures, if any

"B" Sample Confirmation Results
- "B" sample container chain(s) of custody
- Summary of the analytical principles of the confirmation method (if different than "A")
- Aliquot chain of custody
- Sequence verification data
- Confirmation procedure data, including chromatograms (or other relevant data), for
 - Negative control urine
 - Positive control urine (with concentration indicated, if relevant)
 - Standard(s)/calibrator(s) (if relevant)
 - Sample urine aliquot(s)
 - Analytical run instrument validation data (e.g., tune data)
- "B" sample report (including numerical data for threshold substances*)
- Documentation of any deviations from the written screening procedures, if any

Reports and Correspondence
- All facsimiles or letters related to analysis and reporting of sample results

*For threshold substances, an estimate of the ratio or concentration or an estimate of the concentration relative to the threshold i.e. 20 times the threshold concentration) is deemed acceptable.

Johncie Wingard

From: Varin Christian - UCI [christian.varin@uci.ch]
Sent: Tuesday, August 08, 2006 5:52 AM
To: email direction LNDD
Cc: Travis Tygart
Subject: FW: USADA and UCI request

Importance: High

Attachments: 20060808131811313.pdf



2006080813181131
3.pdf (127 KB)...

Dear Dr. De Ceaurriz,

Both USADA and UCI are aware that the laboratory is now closed for vacations and will reopen in two weeks time!

Nevertheless, we already kindly request that you provide our organizations with full documentation supporting the finding of A and B samples (n° 995474).

We will soon communicate other samples references belonging to the same athlete and we will require the steroid profiles from this athlete!

Waiting for your reply, we thank you for your time and courtesies in this matter!

Yours sincerely,

Christian Varin
Manager Antidopage / Antidoping Manager
Union Cycliste Internationale
CH - 1860 Aigle

Tél : +41-24.468.58.11
Fax : +41-24.468.58.12
www.uci.ch

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

-----Original Message-----
From: scanner@uci.ch [mailto:scanner@uci.ch]
Sent: mardi, 8. août 2006 13:18
To: Varin Christian - UCI
Subject:

Cet e-mail a été envoyé par "RNP918BD2" (LD160).

Date de numérisation: 08.08.2006 13:18:11 (+0200) Contacter si besoin: scanner@uci.ch

ANNEX D

The following documentation will be supplied as the standard documentation package:

Table of contents
List of laboratory staff involved in the test, including signatures and/or initials and position title(s)
Sample identification information
- Organization requesting the test
- Date of sample collection and site identification
- USADA sample identification number
- Laboratory sample identification number

Urine integrity test results (if completed)
Chain of custody documentation for sample container
- Doping Control Notification form (Laboratory copy)
- Transportation chain of custody (e.g., courier documentation, laboratory receipt of container)
- "A" sample container chain(s) of custody
- Documentation of any deviations from the written screening procedures, if any

"A" Sample Screening Results
- Relevant aliquot chain(s) of custody
- Screening procedure data, including chromatograms (or other relevant data), for Negative control urine
 - Positive control urine (with concentration indicated, if relevant)
 - Sample urine aliquot(s)
 - Analytical run instrument validation data (e.g.; tune data)
- Documentation of any deviations from the written screening procedures, if any

"A" Sample Confirmation Results
- Summary of the analytical principles of the confirmation method
- Aliquot chain of custody
- Sequence verification data
- Confirmation procedure data, including chromatograms (or other relevant data), for
 - Negative control urine
 - Positive control urine (with concentration indicated, if relevant)
 - Standard(s)/calibrator(s) (if relevant)
 - Sample urine aliquot(s)
 - Analytical run instrument validation data (e.g.; tune data)
- "A" sample report (including numerical data for threshold substances*)
 - pH, Specific Gravity, and other urine integrity test results (if applicable, including abnormal appearance of sample) performed in laboratory.
- Documentation of any deviations from the written screening procedures, if any

"B" Sample Confirmation Results
- "B" sample container chain(s) of custody
- Summary of the analytical principles of the confirmation method (if different than "A")
- Aliquot chain of custody
- Sequence verification data
- Confirmation procedure data, including chromatograms (or other relevant data), for
 - Negative control urine
 - Positive control urine (with concentration indicated, if relevant)
 - Standard(s)/calibrator(s) (if relevant)
 - Sample urine aliquot(s)
 - Analytical run instrument validation data (e.g., tune data)
- "B" sample report (including numerical data for threshold substances*)
- Documentation of any deviations from the written screening procedures, if any

Reports and Correspondence
- All facsimiles or letters related to analysis and reporting of sample results

*For threshold substances, an estimate of the ratio or concentration or an estimate of the concentration relative to the threshold i.e. 20 times the threshold concentration) is deemed acceptable.

Johncie Wingard

From: Varin Christian - UCI [christian.varin@uci.ch]
Sent: Wednesday, August 09, 2006 7:39 AM
To: Travis Tygart
Subject: RE: documentation package for A sample n°995474

Travis,

According to my information, the lab will reopen on 21st August 2006. We may consider that "b" pack will not be sent before this date.

We will send the "a" pack by Friday 11th August 2006!

Thank you and all USADA staff for your very high professional standards in anti-doping fight!

Regards!

Christian

-----Original Message-----
From: Travis Tygart [mailto:ttygart@usantidoping.org]
Sent: mercredi, 9. août 2006 15:16
To: Varin Christian - UCI
Subject: Re: documentation package for A sample n°995474

Please forward is a copy and we will ensure the athlete receives it as well. I believe Caroline was shown the pack along with the others at the lab but my understanding is that she did not receive a copy.

We look forward to receiving it directly from you. Any idea when the "b" pack will be sent?

Thanks for your professionalism.

Travis

Sent from my BlackBerry Wireless Handheld

-----Original Message-----
From: Varin Christian - UCI <christian.varin@uci.ch>
To: Travis Tygart <ttygart@usantidoping.org>
Sent: Wed Aug 09 06:30:51 2006
Subject: documentation package for A sample n°995474

Dear Travis,

I received this morning a copy of the documentation package for A sample.

According to my information, Carolyn has already received a copy from he laboratory when she was in Paris!

Would you like however that I send you a copy?

Thank you for your time and courtesies in this matter!

Best regards!

Christian Varin

Manager Antidopage / Antidoping Manager

Union Cycliste Internationale

CH - 1860 Aigle

Tél : +41-24.468.58.11

Fax : +41-24.468.58.12

www.uci.ch <http://www.uci.ch/>

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, the 23rd August 2006

TRANSMISSION DE TELECOPIE

Expéditeur :	Destinataire :
J. de CEAURRIZ Directeur du Laboratoire National de Dépistage du Dopage Tél : +33 (0) 1.46.60.28.69 Fax : +33 (0) 1.46.60.30.17 e-mail : direction@lndd.com	Travis T. Tygart Organisme : USADA Fax : 00.1.719.785.2001

Nombre de pages y compris celle-ci : 1

Dear Travis,

This fax letter is to announce our sending of the full documentation package related to the counter-analysis of sample B 995474 (139 pages). This sending is made today to USADA by express mail (International Chronopost).

Requests and comments from Dr. DE BOER were annexed to the full documentation package (pages 137 to 139). Please, examine them attentively and inform me about any additional information you would like to receive in this respect.

According to the e-mail from UCI (August 8th, 2006), we are waiting communication of other sample references belonging to the same athlete in order to establish the steroid profiles from this athlete. This additional information will be sent as soon as possible to USADA.

Yours Sincerely,

J. de CEAURRIZ

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

From: email direction LNDD [mailto:direction@lndd.com]
Sent: Tuesday, August 01, 2006 3:47 AM
To: christian.varin@uci.ch; USADA Communications; im.buxeda@mbm-abogados.com
Subject: Contre analyse Landis

This e-mail is to inform you that the counter-analysis of sample B 995474 will start on Thursday August 3 at 9 a.m in the LNDD and will conclude on Saturday, August 5, 2006.
Please note that the athlete will be represented by Mr José M. BUXEDA and Dr Douwe de Boer, USADA by Caroline HATTON and UCI by Dr Martial SAUGY.

Sincerely,

J. de CEAURRIZ

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

August 1, 2006

Prof. Jacques de Ceaurriz, Director
Laboratoire National de Depistage du Dopage
143, avenue Roger Salengro
92290 Chatenay-Malabry
FRANCE

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

RE: UCI File No. 29/06
Tour de France, July 20, 2006
Floyd Landis – Sample #995474

Dear Prof. Ceaurriz:

Please be advised that the United States Anti-Doping Agency ("USADA") has authorized Caroline Hatton, Ph.D., to be its representative at the B sample opening and analysis in the above referenced matter.

If you have any questions or need any additional information about this, please call me immediately at my office at (719)785-2032, on my cell at (719)238-3075 or at my home at (719)635-7264.

Sincerely,

Travis T. Tygart
General Counsel

Johncie Wingard

om: Lyndsey Albright on behalf of USADA Communications
Sent: Thursday, August 24, 2006 7:54 AM
To: Travis Tygart
Subject: FW: For M. TYGART - Steroids profiles
Attachments: Profils stéroïdien sample 995474.doc

Lyndsey Albright
Communications and Legal Affairs Coordinator
U.S. Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(719)785-2011
http://www.usantidoping.org

From: email direction LNDD [mailto:direction@lndd.com]
Sent: Thursday, August 24, 2006 4:55 AM
To: USADA Communications; 'Varin Christian - UCI'; Martial.Saugy@chuv.ch; p.dautry@cpld.fr
Cc: Corinne; Cynthia
S' 'ect: For M. TYGART - Steroids profiles

Dear Travis,

We received yesterday from UCI other sample references belonging to the same athlete as n° 995474. Please, find here a summary of the steroid profiles of this athlete.

My opinion is that sample A 995474 that was collected on July 20th 2006 and declared positive for Testosterone by our laboratory may be regarded as highly suspicious on the basis of the Testosterone, Androsterone, Etiocholanolone, 5α and 5β-Androstanediol levels as well as on the basis of the T/E ratio value.

Sample A 994276 which was collected on July, 14th 2006 may also be regarded as a suspicious sample mainly on the basis of the 5α-Androstanediol, 5β-Androstanediol and Etiocholanolone levels.

The laboratory documentation in relation with the steroid profile data will be sent to USADA and UCI next week by express mail.

Sincerely yours,

J. de CEAURRIZ
LNDD
143 avenue Roger Salengro
92290 CHATENAY-MALABRY
France

Tél : +33.1.46.60.28.69
Fax : +33.1.46.60.30.17
e-mail : direction@lndd.com

STEROID PROFILES RELATED TO SAMPLE 995474 (Tour de France 2006)

	Testo Scr		Epi Scr		T/E Scr	Andro Scr		Etio Scr		5α-diol		5β-diol		δ
	C	Ca	C	Ca		C	Ca	C	Ca	C	Ca	C	Ca	
19/07_995462 03/07/2006	12	9.5	4	3.2	2.8	686	549	992	794	17	11.2	89	71	1.025
99/07_994203 11/07/2006	17	15.5	15	13.7	1.3	1210	1101	989	900	20	18.2	80	73	1.022
121/07_994277 13/07/2006	24	16.6	11	7.6	2.5	1842	1271	1321	911	55	38	100	96	1.029
128/07_994276 14/07/2006	21	16.2	17	13.1	1.5	1594	1227	2041	1572	85	65	333	256	1.026
151/07_994075 15/07/2006	22	20	13	11.8	1.8	1017	925	823	749	27	24.6	71	65	1.022
178/07_995474 20/07/2006	61	47	14	10.8	4.9	1658	1277	1639	1262	97	74.7	227	175	1.026
188/07_994080 22/07/2006	23	20	11	9.6	2.5	919	800	771	670	39	33.9	94	82	1.023
193/07_994171 23/07/2006	11	8.5	12	9.2	1.0	802	618	758	584	17	13.1	51	39	1.026

δ = Specific gravity

Ca = Concentration adjusted for specific gravity (ng/mL)

C = Concentration without adjustment for specific gravity (ng/mL)

USADA 0405

LABORATOIRE NATIONAL
DE DEPISTAGE DU DOPAGE

Châtenay-Malabry the 29th of August

Dear Travis,

As announced in our last email, please find here enclosed the documentation of the steroid profiles related to N°995474 (Tour de France 2006).

Sincerely yours,

J. de CEAURRIZ

Directeur Général

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

STEROID PROFILE RELATED
TO SAMPLE 995474
TOUR DE France 2006

USADA 0407

TABLE OF CONTENT

1 – Summary of the steroid profiles	Page 1
2 – Sample 19/07 – 995462	
2-1 Antidoping control form	Page 2
2-2 Laboratory form as a summary of the follow up of the analytical results	Pages 3 and 4
2-3 Screening results for native compounds involved in the steroid profile	Pages 5 to 7
3 – Sample 99/07 – 994203	
3-1 Antidoping control form	Page 8
3-2 Laboratory form as a summary of the follow up of the analytical results	Pages 9 and 10
3-3 Screening results for native compounds involved in the steroid profile	Pages 11 to 13
4 – Sample 121/07 – 994277	
4-1 Antidoping control form	Page 14
4-2 Laboratory form as a summary of the follow up of the analytical results	Pages 15 and 16
4-3 Screening results for native compounds involved in the steroid profile	Pages 17 to 19
5 – Sample 128/07 - 994276	
5-1 Antidoping control form	Page 20
5-2 Laboratory form as a summary of the follow up of the analytical results	Pages 21 and 22
5-3 Screening results for native compounds involved in the steroid profile	Pages 23 to 25
6 – Sample 151/07 – 994075	
6-1 Antidoping control form	Page 26
6-2 Laboratory form as a summary of the follow up of the analytical results	Pages 27 and 28
6-3 Screening results for native compounds involved in the steroid profile	Pages 29 to 31
7 – Sample 178/07 – 995474	
7-1 Antidoping control form	Page 32
7-2 Laboratory form as a summary of the follow up of the analytical results	Pages 33 to 35
7-3 Screening results for native compounds involved in the steroid profile	Pages 36 to 41
8 – Sample 188/07 – 994080	
8-1 Antidoping control form	Page 42
8-2 Laboratory form as a summary of the follow up of the analytical results	Pages 43 and 44
8-3 Screening results for native compounds involved in the steroid profile	Pages 45 to 47
9 – Sample 193/07 – 994171	
9-1 Antidoping control form	Page 48
9-2 Laboratory form as a summary of the follow up of the analytical results	Pages 49 and 50
9-3 Screening results for native compounds involved in the steroid profile	Pages 51 to 53

Summary of the steroid profiles

STEROID PROFILES RELATED TO SAMPLE 995474 (Tour de France 2006)

	Testo Scr		Epi Scr		T/E Scr	Andro Scr		Etio Scr		5α-diol Scr		5β-diol Scr		δ
	C	Ca	C	Ca		C	Ca	C	Ca	C	Ca	C	Ca	
19/07_995462 03/07/2006	12	9.5	4	3.2	2.8	686	549	992	794	17	11.2	89	71	1.025
99/07_994203 11/07/2006	17	15.5	15	13.7	1.3	1210	1101	989	900	20	18.2	80	73	1.022
121/07_994277 13/07/2006	24	16.6	11	7.6	2.5	1842	1271	1321	911	55	38	100	69	1.029
128/07_994276 14/07/2006	21	16.2	17	13.1	1.5	1594	1227	2041	1572	85	65	333	256	1.026
151/07_994075 15/07/2006	22	20	13	11.8	1.8	1017	925	823	749	27	24.6	71	65	1.022
178/07_995474 20/07/2006	61	47	14	10.8	4.9	1658*	1277*	1639*	1262*	97	74.7	227	175	1.026
188/07_994080 22/07/2006	23	20	11	9.6	2.5	919	800	771	670	39	33.9	94	82	1.023
193/07_994171 23/07/2006	11	8.5	12	9.2	1.0	802	618	758	584	17	13.1	51	39	1.026

* * Results obtained by analysis of an aliquot from the confirmation step using the screening analysis procedure due to inhibition of the derivatisation of andro and etio in the screening step.

δ = Specific gravity

Ca = Concentration adjusted for specific gravity (ng/mL)

C = Concentration without adjustment for specific gravity (ng/mL)

USADA 0410

Sample 19/07 – 995462

2 – LABORATOIRE
ÉCHANTILLONS A + B
NFIDENTIEL

Union Cycliste Internationale

UCI 2006

RLINGER

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTR 19.07
Test certificate

Contrôle urinaire / Urine test ☒
Contrôle sanguin / Blood test ☐

1. En compétition / In competition ☒
 Hors compétition / Out of competition ☐
2. Sexe: Masculin / Male ☒
 Sex: Féminin / Female ☐
3. Date: 3/07/2006
4. Lieu / Place: ESCH SUR ALZETTE
5. Course (nom, étape):
 Race (name, stage): TOUR DE FRANCE 2ème ETAPE
6. Discipline:
 Discipline: ROUTE G.T.

[redacted]

12. Heure de présentation:
 Presentation time: 17,25
13. Heure de prélèvement:
 Time of sampling: 17,30

Volume _____ pH* _____ Densité / Specific gravity* _____
*Optionnel / Optional

14. Code des flacons:
 Bottle codes: BUR995462
15. Heure de refus du coureur:
 Time of rider's refusal:

16. Médicaments pris:
 Pharmaceutical drugs taken by rider: _____

Contenu du livret de santé:
Contents of the health booklet:
5.05.06 Decapeptyl 3 Inj i.m. Local 6.8 Wk AUT
5.05.06 Methylprednisolon 5 Inj i.m. Local 6-8 Wk AUT

17. Autorisation d'usage à des fins thérapeutiques (AUT)
 Therapeutic use exemption (TUE)
 Oui / Yes ☒
 Non / No ☐

[redacted]

20. Médecin contrôleur:
 Examining doctor: BORDABERRY GERARD (nom/name) (signature)

Agent de prélèvement sanguin:
Blood collection officer: _____ (nom/name) _____ (signature)

1. Inspecteur antidopage:
 Antidoping inspector: MERAVIGLIA GIOVANNI (nom/name) Meraviglia Giovanni (signature)

ATTENTION LABORATOIRES: Le rapport d'analyse est à envoyer à: **Union Cycliste Internationale**
CH-1860 Aigle Fax +41 24 468 59 14

USADA 0412

LNDD	ENREGISTREMENT FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS	Codification : E-Fiche_Labo-01 Version : Q Date : 18/05/2006 Page : 1 / 2

Numéro Labo: 19/07 Médecin BORDABERRY Gérard Matériel: Berlinger Nbre 4

Sport Cyclisme (UCI) En compet (I)

Epreuve (F.I.) TDF 2006 2ème étape : Esch-sur-Alzette

Date(s) de Prél.: 03/07/2006 Récept° Ech. : 03/07/2006 Récept° Doc. : 03/07/2006

Date de mise en tube : 04/07/06 Date MT EPO : 04/07/06 Date MT HES : 04/07/06

Observation PV UCI
Délai 48H EPO/HES

Ecarts: EA n°: Réclamat° N° :

N° :	995379	995386	995429	995462								Operateur
Sexe:	M	M	M	M								V20
Flacon (F)												Operateur
volume	80	80	>100	70								42
pH-bande	6	6	6	5								42
d-refract	1,038	1,024	1,017	1,025								42
ES06-Im	X	X	X	X								
ES02-Eph	X	X	X	X								
ES02-bbs	X	X	X	X								
ES04-H	X	X	X	X								
ES07-ETg/ETg	0	2,3	2,0	2,8								
ES05-MS2	X	X	X	X								
ES03-cd	X	X	X	X								
ES03B-LCH	X	X	X	X								
ES08B-PS	X	X	X	X								
ES08-HES	X	X	X	X								
ES07-Epo	X	X	X	X								
ES01-HBO												
ES02-TS												

Résultats :

995379 : Salbutamol < seuil

Vérifié par : [illegible] Date : 04/07/06

A et B accouplés : le : par :

A et B détruits: le : par :

USADA 0413

LNDD	Enregistrement	Codification : E-Fiche_Labo-01 Version : 0 Date 18/05/2006 Page : 2/2

FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS

CONCLUSIONS

Essai	Echantillon	RESULTATS ANORMAUX ET RESERVES	Date et Visa
ES		Présence de pH : d : (+/-) Dépistage : Conf :	
ES		Présence de pH : d : (+/-) Dépistage : Conf :	
ES		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH : d : (+/-) Dépistage : Conf :	
ES		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH : d : (+/-) Dépistage : Conf :	
S 01	995329	NB : les résultats de(s) l'essai(s) ES 02 ne sont pas totalement exploitables en raison d'une inhibition de l'hydrolyse due à une interférence matricielle	5/7/6
S03	995329 995326	NB les résultats de l'essai ES03 ne sont pas totalement exploitables en raison d'une inhibition de l'extraction due à une interférence matricielle	06/07/06 AL

Si la place est insuffisante pour conclure, utiliser E-Fiche_Labo-01Bis en complément ; dans ce cas, indiquer le nombre de feuilles utilisées :

RESULTATS NORMAUX	Date et Visa	RESULTATS NORMAUX	Date et Visa
Recherche négative pour les essais ES01, ES02 et ES04 et ES05 ES02 OK	5/7/6 M Ecart :	IMM RESULTAT DANS LES NORMES POUR L'ESSAI ES06	050706 NB Ecart :
		EPO Absence d'EPO recombinante ES 01	6/7/06 M Ecart :
Recherche négative pour les essais ES03 et ES03B (HA)	06/07/06 AL Ecart :	HES COCO Recherche d'HEA négative par ES08B	6/7/6 M Ecart :

Edition du (des) rapport(s) d'analyse : Visa : CL

Vérification du(des) rapport(s) d'analyse par la LT et l'AQ Conv Date et Visa : AL 06/07/06

USADA 0414

5

D:\MSD23\JUIL07\0407\04h08.D

Data File Path D:\MSD23\JUIL07\0407\
Data File Name **04h08.D**
Operator **M23P24**
Acq. Method File **MAN06B_B.M**
Sample Name **19/07 995462 H**
Misc Info
Vial Number **11**
Instrument Name **msd23**
Calibration Title **Calibration des stéroides endogènes**
Last Calibration Update **Tue Jul 04 10:43:31 2006**
Fichier recal utilise **04rcl01.D**

Date : 05/07/06
Opérateur : ..16..

C [] HC []

Résultats : Négatif [M]
A vérifier []

Remarques :
.....
.....

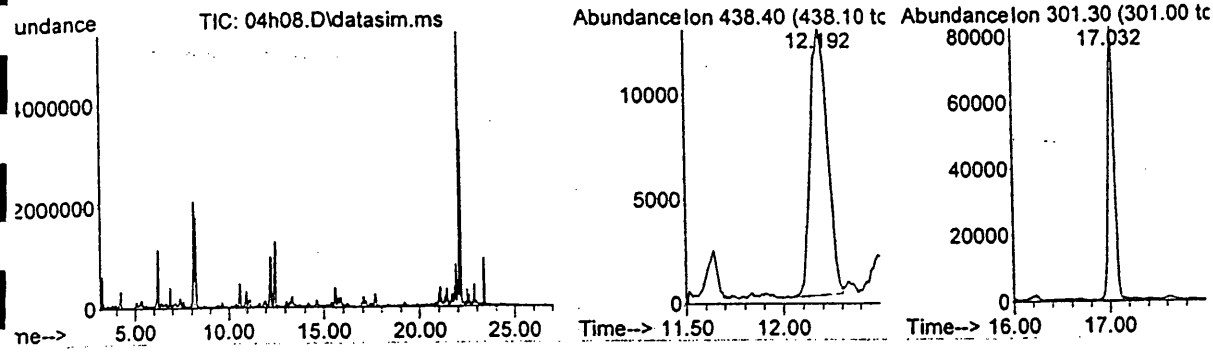
#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	17.03	301.3	Methyltestosterone	3,527,508	200	ng/ml
2)		12.19	438.4	Andro -D4 gluc	779,404	82	ng/ml
3)		4.14	372.3	Salbutamol -D3	2,168,879	180	ng/ml
4)		12.26	434.4	Androsterone	12,115,805	686	ng/ml
5)		12.52	434.4	Etiocholanolone	18,752,285	992	ng/ml
6)		12.65	241.2	5a3a diol	129,770	14	ng/ml
7)		12.82	241.2	5b3a diol	765,306	89	ng/ml
8)		17.67	117.1	Pregnanediol	8,543,363	197	ng/ml
9)		0.00	434.4	DHT	0	0	ng/ml
10)		13.72	432.4	DHEA	247,036	15	ng/ml
11)		14.27	432.4	Epitestosterone	221,520	4	ng/ml
12)		15.14	432.4	Testosterone	611,340	12	ng/ml
13)		17.57	371.3	THC -M	62,069	14	ng/ml
14)		0.00	369.3	Salbutamol	0	-45	ng/ml
15)		15.88	522.5	11 bOH Etiocholanolone	3,415,009	215	ng/ml
16)		21.31	548.5	THS	2,350,612	44	ng/ml
17)		22.08	636.6	THF	79,075,585	1067	ng/ml
18)		22.82	632.6	Cortisol	2,525,757	66	ng/ml
19)		14.62	343.3	11 Ketoetiocholanonlone	2,943,198	216	ng/ml
20)		10.06	432.4	3a-5C	2,072		ng/ml

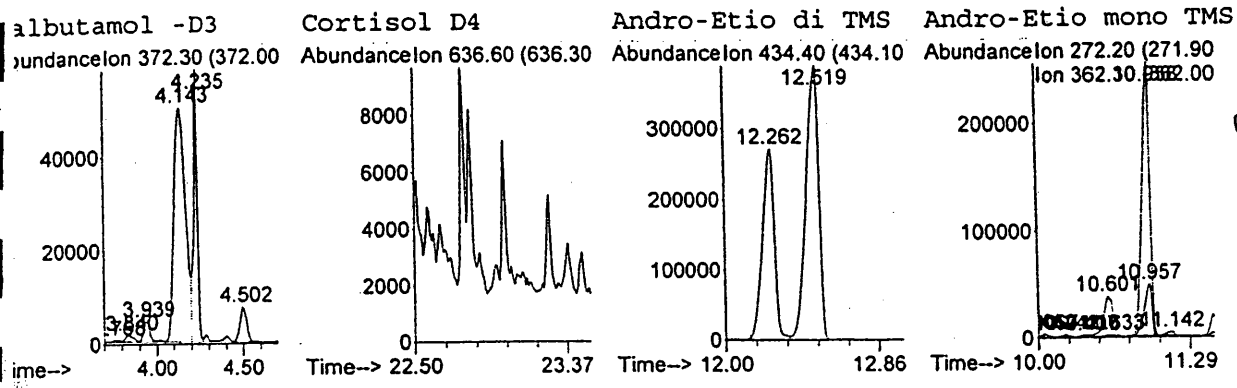
	[Surface]	[concentration]	Valeurs conventionnelles de rejet	
Ad4 glu / Si *100	22.1			< 15.7
T / ET	2.8		Rapport	> 4
Testosterone		11.5	Concentration	> 200
Epitestosterone		4.3	Concentration	> 200
Androsterone		686	Concentration	> 10000
Etiocholanolone		992	Concentration	> 10000
DHEA		15	Concentration	> 200
DHT		0	Concentration	> 20
Andro / Etio	0.6		Rapport	> 3
5a3a / 5b3a diol	0.2		Rapport	> 2
THC -M		14.2	Concentration	> 15
Salb/Salb D3 * 100	0	-45.0	Concentration	> 75
Cortisol / THS		1.5		
THF / THS		24.3		
11 bOH etio / THS		4.9	Pour info (R&D)	
THF		1067		
SI/3a5c	1702.2			

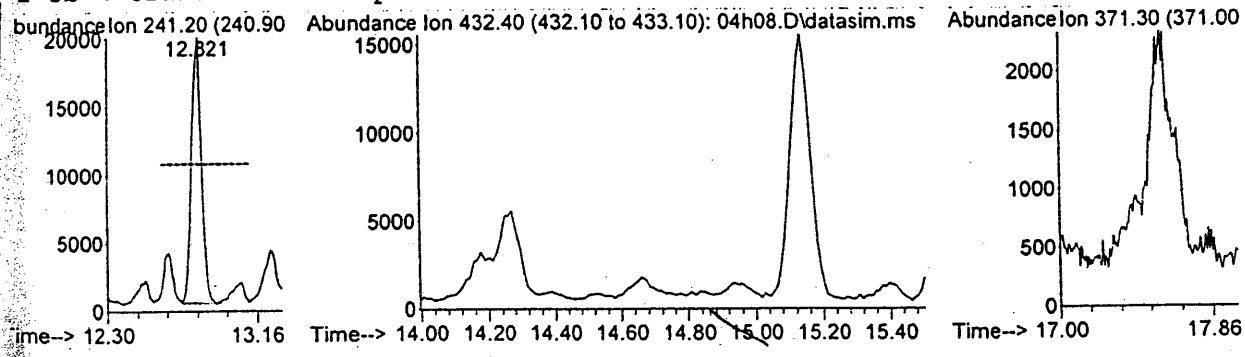
D:\MSD23\...

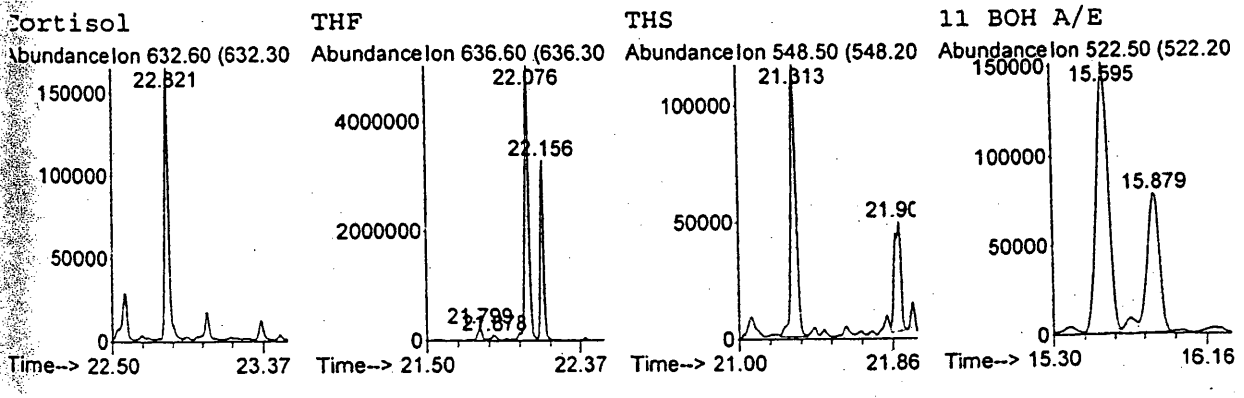
cor: M23P24
Acquired: 4 Jul 06 8:00 pm
strument: msd23
ethod File: MAN06B_B.M
Sample Name: 19/07 995462 H
Misc Info:
Vial Number : 11

6





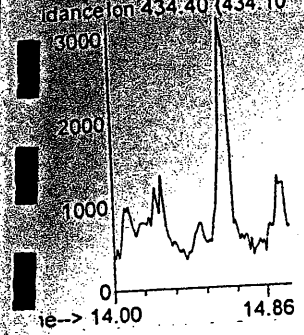


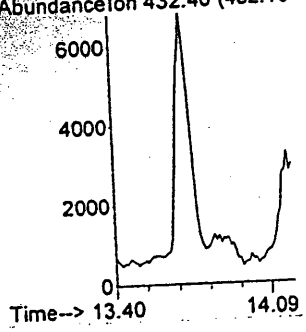


USADA 0416

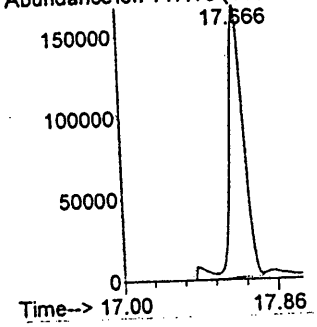
:or: M23P24
Acquired: 4 Jul 06 8:00 pm
trument: msd23
thod File: MAN06B_B.M
ample Name: 19/07 995462 H
ise Info:
al Number : 11

DHEA

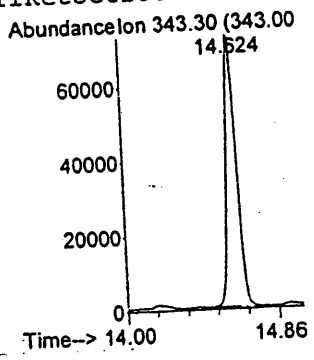




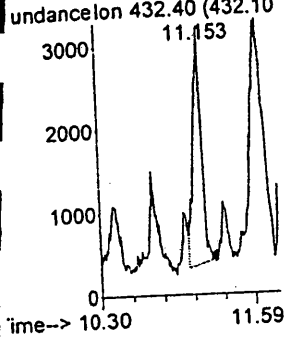
Prégnanediol



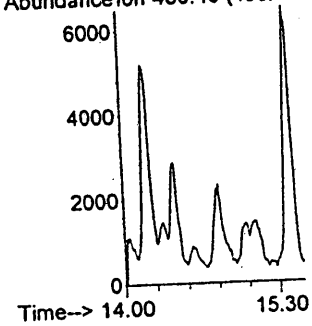
11Ketoetiocholanolone



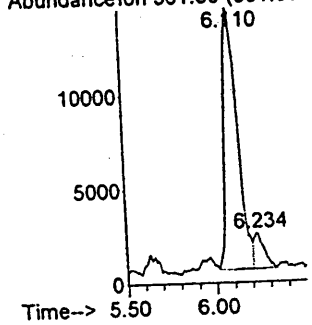
Androstandione



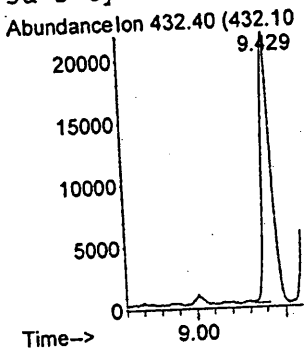
4 Androstenedione



Inhib derivation



3a-5 Cyclo



Sample 99/07 - 994203

8

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM

CONFIDENTIEL / CONFIDENTIAL

2006
91.07

Organisme demandant le contrôle / Organization requesting the test

☐ Ministère chargé des Sports
☐ Direction régionale de la Jeunesse et des Sports
☐ Fédération nationale
☒ Fédération internationale

Type de contrôle / Type of test

Inopiné / Out ☐ Non ☒

Compétition	Hors Compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Stade ☐

Sexe / Sex: M ☐ F ☐

Fédération - Discipline / Sport

Nom de l'épreuve - Ville - Pays / Event and place

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date: 11/2/2006

Nom et signature du Délégué Fédéral / Name and Signature of Federal Delegate

Nom et prénom du Médecin / Name of Doctor (en lettres capitales)

Signature du Médecin / Signature of Doctor

Réalisation du contrôle / Test Completion

Date du prélèvement / Sampling date: 11/2/2006

Heure du prélèvement / Sampling time: 17 h 00

Echantillons A et B / Samples A and B
Code flacon et conteneur(s) / Bottle Code

BUR994203

Quantité totale urinée / Total urine volume: 100 ml

Densité / Specific gravity: 1.010

Echantillon d'urine insuffisant / Insufficient urine sample

Médicaments / Drugs ☐ OUI ☒ NON

Médicaments déclarés avoir été récemment / Drugs declared to have been recently used:

Renseignement non du médecin prescripteur

Commentaires sur la procédure / Comments on procedure

Confirmation / Confirmation

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du Médecin / Signature of Doctor

USADA 0419

LNDD	ENREGISTREMENT FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS	Codification : E-Fiche Labo-01 Version : Q Date : 18/05/2006 Page : 1 / 2

Numéro Labo: 99/07 | Médecin: BORDABERRY Gerard | Matériel: Berlinger | Nbre: 3

Sport: Cyclisme (UCI) | En compet.(1)

Epreuve (F.I.): Tour de France 2006 9ème étape à Dax

Date(s) de Prél.: 11/07/2006 | Récept° Ech.: 11/07/2006 | Récept° Doc.: 11/07/2006

Date de mise en tube: 11/07/06 - 00 | Date MT EPO: 12/07/06 | Date MT HES: 12/07/06

Observation: Délai 48h EPO/HES

Ecarts: EA n°: 85+86 | Réclamat° N°:

N° :	994201	994203	995482										Operateur
Sexe:	M	M	M										V22
Filtrat (F)													Operateur
Volume	90	70	70										42
pH-bande	5	5	5										42
d-refract	1,027	1,022	1,021										42
ES06-Im	X	X	X										
ES02-Eph	X	X	X										
ES02-Obs	X	X	X										
S04-H	X	X	X										
IPETG	A	A	A										
ES08-MS2	X	X	X										
ES03-Lev	X	X	X										
ES03B-LCH	X	X	X										
ES08B-PS	X	X	X										
ES08-HES			0,21										
ES07-Epo	X	X	X										
S01-HBC													
ES02-TS													

Résultats :

995482 : Salbu (Sang)

994203 : Efedrina (Urine)

Vérifié par : Date :

A et B accouplés : le : par :

et B détruits: le : par :

LNDD	Enregistrement	Codification : E-Fiche Labo-01 Version : Q Date 18/05/2006 Page : 2/2

FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS

CONCLUSIONS

Essai	Echantillon	RESULTATS ANORMAUX ET RESERVES	Date et Visa
ES...		Présence de pH : d : (+/-) Dépistage : Conf :	
ES...		Présence de pH : d : (+/- %) Dépistage : Conf :	
S ...		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH : d : (+/-) Dépistage : Conf :	
S ...		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH : d : (+/-) Dépistage : Conf :	
S ...		NB : les résultats de(s) l'essai(s) ES ne sont pas totalement exploitables en raison d'une inhibition de due à une interférence matricielle.	

Si la place est insuffisante pour conclure, utiliser E-Fiche Labo-01 Bis en complément dans ce cas, indiquer le nombre de feuilles utilisées :

RESULTATS NORMAUX	Date et Visa	RESULTATS NORMAUX	Date et Visa
C Recherches négatives par les essais ES02, ES02C*, ES04 et ES05	B10706 Ecart :	IMM RESULTAT DANS LES NORMES POUR L'ESSAI ES06	130106 Ecart :
		EPO Absence d'EPO endogènes par l'essai ES07	140106 Ecart :
C Recherche négative pour les essais ES03 et ES03B (HA)	13/07/06 Ecart : ES RAS	~~HES~~ HEA Recherche négative par ES08B* négative	13/07/06 Ecart :

Edition du (des) rapport(s) d'analyse : Visa :

Vérification du(des) rapport(s) d'analyse par la LT et l'AQ ☐ Conv ☐ Spé Date et Visa : AH 14/07/06

11

D:\MSD23\JUIL07\1207\12h04.D

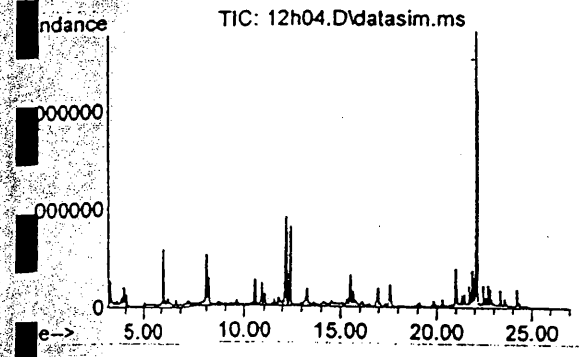
Data File Path D:\MSD23\JUIL07\1207\
Data File Name **12h04.D**
Operator **M23P24**
Acq. Method File **MAN06B_B.M**
Sample Name **99/07 994203 H**
Misc Info
Vial Number **7**
Instrument Name **msd23**
Calibration Title **Calibration des stéroides endogènes**
Last Calibration Update **Wed Jul 12 11:23:01 2006**
Fichier recal utilise **12rcl02.D**

Date : 13/07/06
Opérateur : ...
C [] HC []
Résultats : Négatif / A vérifier
Remarques : ...

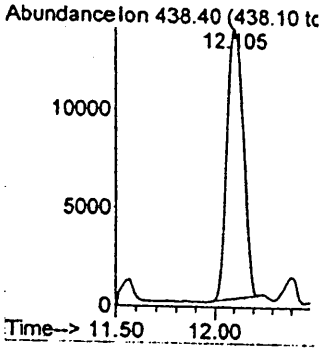
#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	16.95	301.3	Methyltestosterone	3,777,896	200	ng/ml
2)		12.11	438.4	Andro -D4 gluc	824,070	885	ng/ml
3)		4.08	372.3	Salbutamol -D3	1,865,078	140	ng/ml
4)		12.18	434.4	Androsterone	11,031,493	1210	ng/ml
5)		12.41	434.4	Etiocholanolone	10,459,863	989	ng/ml
6)		12.55	241.2	5a3a diol	116,454	20	ng/ml
7)		12.73	241.2	5b3a diol	445,475	80	ng/ml
8)		17.56	117.1	Pregnanediol	9,457,385	277	ng/ml
9)		0.00	434.4	DHT	0	0	ng/ml
10)		13.62	432.4	DHEA	239,427	25	ng/ml
11)		14.18	432.4	Epitestosterone	426,357	15	ng/ml
12)		15.05	432.4	Testosterone	552,337	17	ng/ml
13)		17.47	371.3	THC -M	25,392	2	ng/ml
14)		0.00	369.3	Salbutamol	0	-45	ng/ml
15)		15.78	522.5	11 bOH Etiocholanolone	889,911	125	ng/ml
16)		21.28	548.5	THS	1,594,240	47	ng/ml
17)		22.04	636.6	THF	40,286,109	1596	ng/ml
18)		22.79	632.6	Cortisol	2,143,755	99	ng/ml
19)		14.53	343.3	11 Ketoetiocholanonlone	980,559	102	ng/ml
20)		10.10	432.4	3a-5C	12,059		ng/ml

	[Surface]	[concentration]	Valeurs conventionnelles de rejet	
Ad4 glu / Si *100	21.8			**< 15.7**
T / ET	1.3		**Rapport**	**> 4**
Testosterone		17.4	**Concentration**	**> 200**
Epitestosterone		15.3	**Concentration**	**> 200**
Androsterone		1,210	**Concentration**	**> 10000**
Etiocholanolone		989	**Concentration**	**> 10000**
DHEA		25	**Concentration**	**> 200**
DHT		0	**Concentration**	**> 20**
Andro / Etio	1.1		**Rapport**	**> 3**
5a3a / 5b3a diol	0.3		**Rapport**	**> 2**
THC -M		1.8	**Concentration**	**> 15**
Salb/Salb D3 * 100	0	-45.0	**Concentration**	**> 75**
Cortisol / THS		2.1		
THF / THS		34.0		
11 bOH etio / THS		2.7	**Pour info (R&D)**	
THF		1596		
Sl/3a5c	313.3			

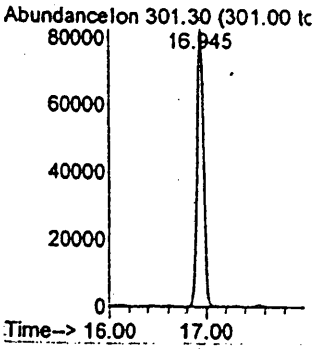
D:\MSD23\JUIL07\1207\12h04.D\da
M23P24
Acquired: 12 Jul 06 5:39 pm
Instrument : msd23
Method File: MAN06B_B.M
Sample Name: 99/07 994203 H
Misc Info:
Vial Number : 7



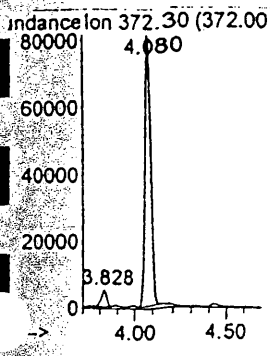
Andro -D4 gluc



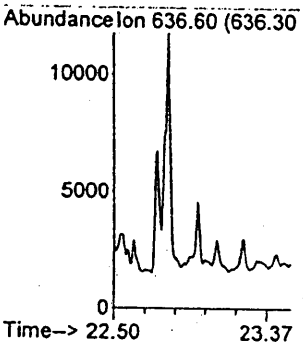
Methyltestosterone



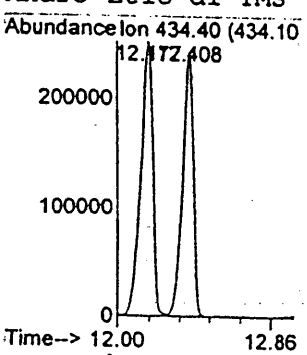
lbutamol -D3



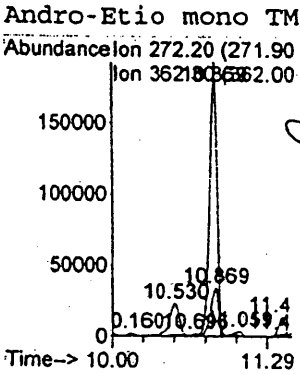
Cortisol D4



Andro-Etio di TMS

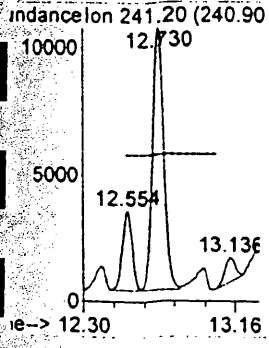


Andro-Etio mono TMS

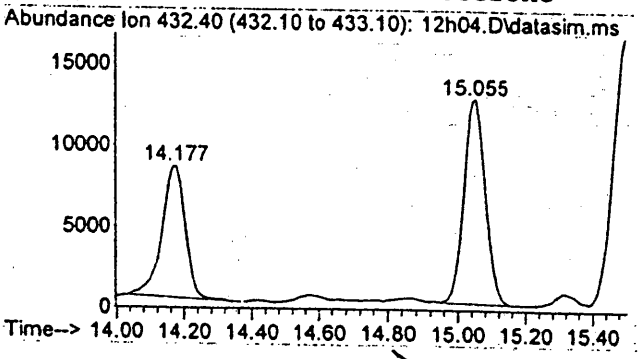


OK
10

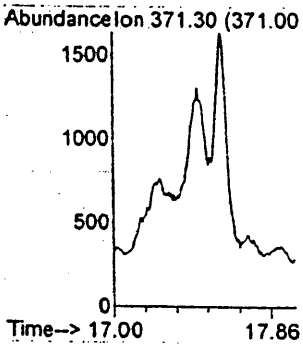
3a-5b3a Diol
5b < trait : IRMS



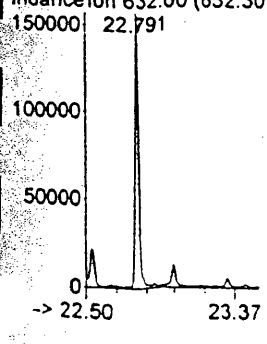
Epitestostérone - Testostérone



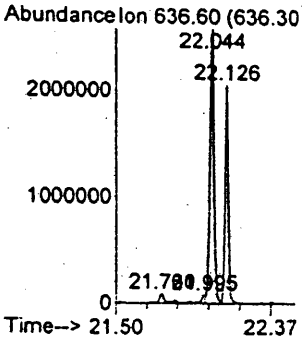
THC -M



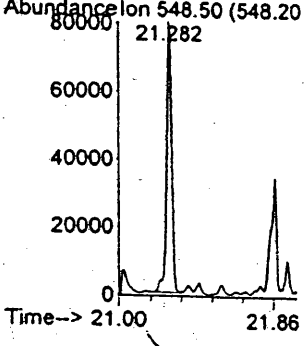
rtisol



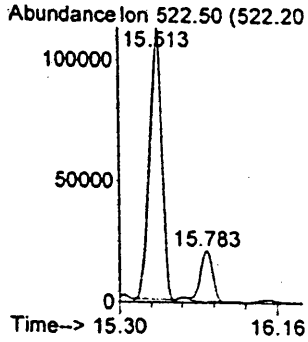
THF



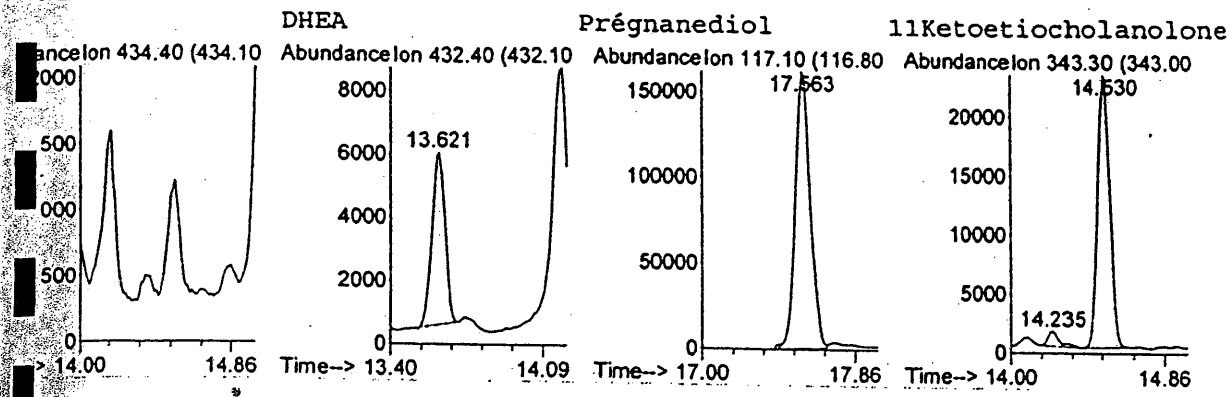
THS

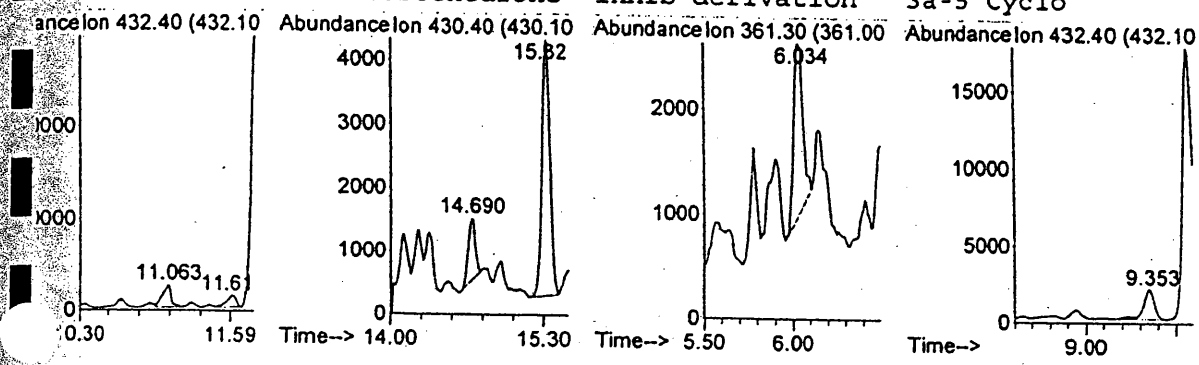


11 BOH A/E



D:\MSD23\JUIL07\1207\12h04_D\da
M23P24
quired: 12 Jul 06 5:39 pm
ment: msd23
d File: MAN06B_B.M
le Name: 99/07 994203 H
c Info:
al Number : 7





Sample 121/07 - 994277

LABORATOIRE CHANTILLONS A + B DENTIEL ERLINGER

Union Cycliste Internationale

UCI 2006

14

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONT
Test certificate

121.07

Contrôle urinaire / Urine test ☒ — Contrôle sanguin / Blood test ☐

1. En compétition / In competition ☒ — Hors compétition / Out of competition ☐
2. Sexe / Sex: Masculin / Male ☒ — Féminin / Female ☐
3. Date: 13/07/2006
4. Lieu / Place: PLA-DE-BERET
5. Course (nom, étape): Race (name, stage): TOUR DE FRANCE 11ème ÉTAPE
6. Discipline: Discipline: ROUTE GRAN TOUR

[redacted]

12. Heure de présentation: Presentation time: 18H00
13. Heure de prélèvement: Time of sampling: 18H20

Volume 100 ml — pH* 5 — Densité / Specific gravity* 1,030

*Optionnel / Optional

14. Code des flacons: Bottle codes: BUR994277
15. Heure de refus du coureur: Time of rider's refusal:

16. Médicaments pris: Pharmaceutical drugs taken by rider:

Contenu du livret de santé: Contents of the health booklet:
5.5.06 Deraxethane / Local 6-8 week
05.05.06 Methylprednisolone Local 6-8 Week
8.7.06 Deraxethane / Injection 2 wk
8/7/06 Methylprednisolone 2 wk

AVT

17. Autorisation d'usage à des fins thérapeutiques (AUT) Therapeutic use exemption (TUE) — Oui / Yes ☒ — Non / No ☐

[redacted]

20. Médecin contrôleur: Examining doctor: BORDABERRY GERARD (nom/name) (signature)

Agent de prélèvement sanguin: Blood collection officer: (nom/name) (signature)

Inspecteur antidopage: Antidoping inspector: MERAVIGLIA GIOVANNI (nom/name) (signature)

⚠ **ATTENTION LABORATOIRES:** Le rapport d'analyse est à envoyer à: **Union Cycliste Internationale**
ATTENTION LABORATORIES: The analyse-report is to send to: CH-1860 Aigle Fax +41 24 468

USADA 0426

LNDD	ENREGISTREMENT FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS	Codification : E-Fiche_Labo-01 Version : Q Date : 18/05/2006 Page : 1 / 2

Numéro Labo: 121/07 Médecin BORDABERRY Gerard Matériel: Berlinger Nbre 4

Sport Cyclisme UCI En compétition

Epreuve (F.I.) TDF 2006 étape n° 11 : Ria de Berat

Date(s) de Prél.: 13/07/2006 Récept° Ech. : 14/07/2006 Récept° Doc. : 14/07/2006

Date de mise en tube : [illegible] Date MT EPO : [illegible] Date MT HES : [illegible]

Observation délai 48h P.V UCI

EPO/HES

Ecarts: EA n°: 0 Réclamat° N°:

N° :	994268	994269	994274	994277								Operateur
Sexe:	M	M	M	M								V20
Filtral (F)				F								Operateur
Volume :	95	95	>100	65								44
pH-bande	6	5	5	5								44
d-refract	1,024	1,022	1,025	1,029								44
ES08-Im	X	X	X	X								
ES02-Eph	X	X	X	X								
ES02-bcs	X	X	X	X								
ES04-HI	X	X	X	X								
ES04-eTg	0,4	0,9	1,2	2,5								
ES05-MS2												
ES03-cd	X	X	X	X								
ES03B-LCH	X	X	X	X								
ES08B-PS	X	X	X	X								
ES08-HES												
ES07-Epo	X	X	X	X								
ES01-HBO												
ES02-TS												

Résultats :

994274 TA < limite ne pas signer NL

Vérifié par : [illegible] Date : 19/7/06

A et B accouplés : le : par :

B détruits: le : 8/10 par :

LNDD	Enregistrement	Codification : E-Fiche_Labo-01 Version : Q Date 18/05/2006 Page : 2/2

FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS

CONCLUSIONS

Essai	Echantillon	RESULTATS ANORMAUX ET RESERVES	Date et Visa
ES		Présence de pH : d : (+/-) Dépistage : Conf :	
ES		Présence de pH : d : (+/-) Dépistage : Conf :	
ES		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH : d : (+/-) Dépistage : Conf :	
ES		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH : d : (+/-) Dépistage : Conf :	
ES 02	994277	NB : les résultats de(s) l'essai(s) ES 02 ne sont pas totalement exploitables en raison d'une inhibition de la dilution due à une interférence matricielle	19/7/06

Si la place est insuffisante pour conclure, utiliser E-Fiche_Labo-01Bis en complément ; dans ce cas, indiquer le nombre de feuilles utilisées :

RESULTATS NORMAUX	Date et Visa	RESULTATS NORMAUX	Date et Visa
IC Recherche négative pour les essais ES02C* ; ES02, ES04 et ES05*	19/7/06 Ecart :	IMM RESULTAT DANS LES NORMES POUR L'ESSAI ES06	190706 Ecart :
C		EPO Absence d'EPO recombinante ES07	17/07/06 Ecart :
Recherche négative pour les essais ES03 et ES03B (HA)	16/07/06 AL Ecart :	HES Recherche d'HEA négative pour l'essai ES08B*	19/7/06 Ecart :

Edition du (des) rapport(s) d'analyse : Visa :

Vérification du(des) rapport(s) d'analyse par la LT et l'AQ Conv Spé Date et Visa : 19/07/06

Cet enregistrement est à archiver au département Logistique Technique

17

D:\msd23\juil07\1507\15H05.D

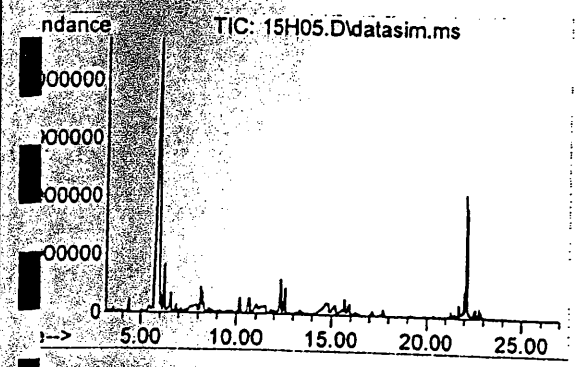
Data File Path D:\msd23\juil07\1507\
Data File Name **15H05.D**
Operator **M18p23/28**
Acq. Method File **MAN06B_B.M**
Sample Name **121/07 994277 H**
Misc Info
Vial Number **8**
Instrument Name **msd23**
Calibration Title **Calibration des stéroides endogènes**
Last Calibration Update **Sat Jul 15 10:18:12 2006**
Fichier recal utilise **15rcl01.D**

Date : 18/07/06
Opérateur : 10
C [] HC []
Résultats : Négatif [X]
A vérifier []
Remarques : Inhibiteur
..... de dérivation
..... 09

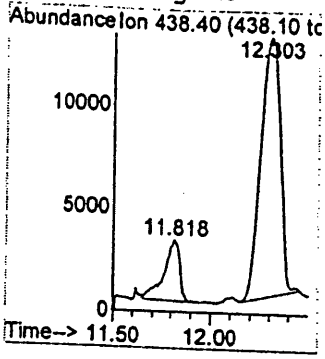
#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	17.11	301.3	Methyltestosterone	3,884,192	200	ng/ml
2)		12.30	438.4	Andro -D4 gluc	948,365	1111	ng/ml
3)		4.17	372.3	Salbutamol -D3	1,381,140	113	ng/ml
4)		12.36	434.4	Androsterone	15,944,469	1842	ng/ml
5)		12.60	434.4	Etiocholanolone	13,445,500	1321	ng/ml
6)		12.73	241.2	5a3a diol	299,884	55	ng/ml
7)		12.90	241.2	5b3a diol	529,871	100	ng/ml
8)		17.71	117.1	Pregnanediol	1,016,159	44	ng/ml
9)		0.00	434.4	DHT	0	0	ng/ml
10)		13.74	432.4	DHEA	322,716	34	ng/ml
11)		14.28	432.4	Epitestosterone	299,437	11	ng/ml
12)		15.27	432.4	Testosterone	739,856	24	ng/ml
13)		0.00	371.3	THC -M	0	0	ng/ml
14)		0.00	369.3	Salbutamol	0	-45	ng/ml
15)		15.69	522.5	11 bOH Etiocholanolone	8,716,209	1751	ng/ml
16)		21.30	548.5	THS	2,730,141	92	ng/ml
17)		22.07	636.6	THF	73,294,838	3359	ng/ml
18)		22.80	632.6	Cortisol	4,054,767	322	ng/ml
19)		14.44	343.3	11 Ketoetiocholanonlone	153,703	17	ng/ml
20)		10.20	432.4	3a-5C	29,001		ng/ml

	[Surface]	[concentration]	Valeurs conventionnelles de rejet	
Ad4 glu / Si *100	24.4			**< 15.7**
T / ET	2.5		**Rapport**	**> 4**
Testosterone		24.3	**Concentration**	**> 200**
Epitestosterone		11.4	**Concentration**	**> 200**
Androsterone		1,842	**Concentration**	**> 10000**
Etiocholanolone		1,321	**Concentration**	**> 10000**
DHEA		34	**Concentration**	**> 200**
DHT		0	**Concentration**	**> 20**
Andro / Etio	1.2		**Rapport**	**> 3**
5a3a / 5b3a diol	0.6		**Rapport**	**> 2**
THC -M		0.0	**Concentration**	**> 15**
Salb/Salb D3 * 100	0	-45.0	**Concentration**	**> 75**
Cortisol / THS		3.5		
THF / THS		36.6		
11 bOH etio / THS		19.1	**Pour info (R&D)**	
THF		3359		
SI/3a5c	133.9			

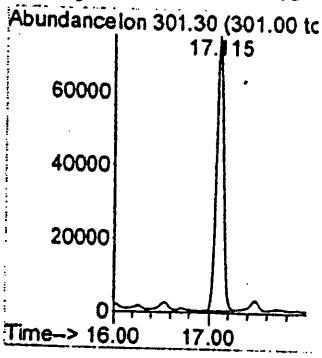
D:\MSD23\JUIL07\1507\15H05.D\da
M18p23/28
Acquired: 15 Jul 06 4:53 pm
Instrument: msd23
Method File: MAN06B_B.M
Sample Name: 121/07 994277 H
Misc Info:
Vial Number : 8



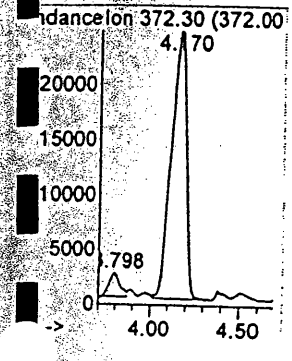
Andro -D4 gluc



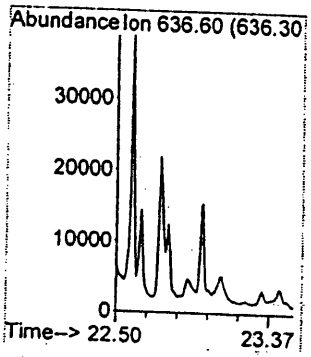
Methyltestosterone



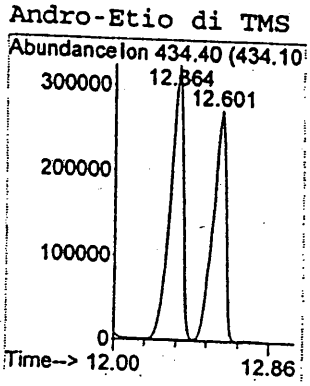
butamol -D3



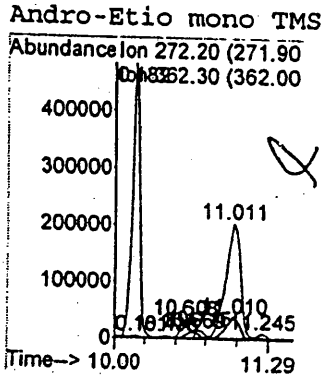
Cortisol D4



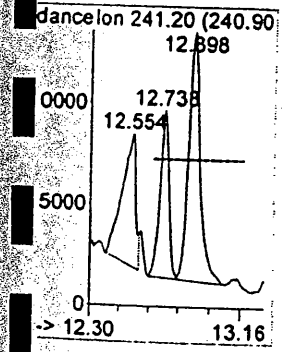
Andro-Etio di TMS

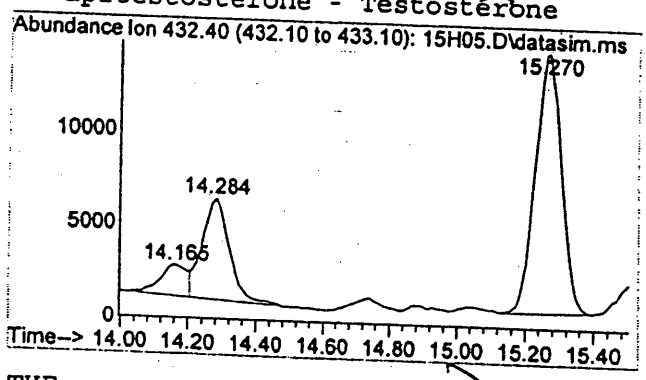


Andro-Etio mono TMS

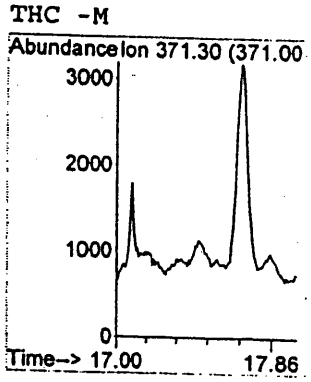


-5b3a Diol
5b < trait : IRMS Epitestostérone - Testostérone

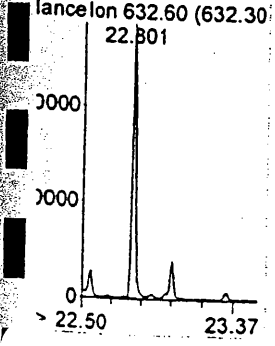




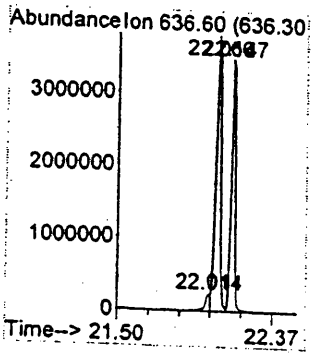
THC -M



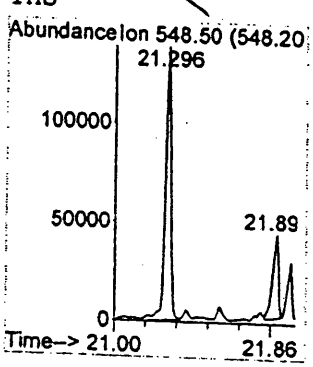
isol



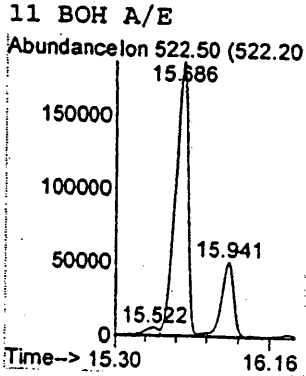
THF



THS



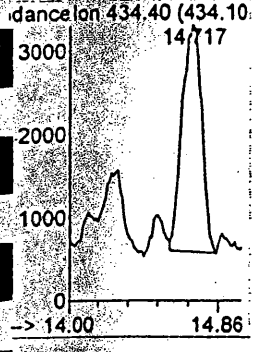
11 BOH A/E

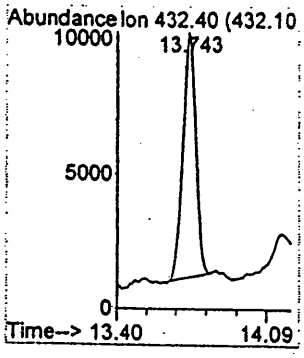


Data File : D:\MSD23\JUIL07\1507\15H05.D\da
Operator: M18p23/28
Date Acquired: 15 Jul 06 4:53 pm
Instrument: msd23
Method File: MAN06B_B.M
Sample Name: 121/07 994277 H
Misc Info:
Vial Number : 8

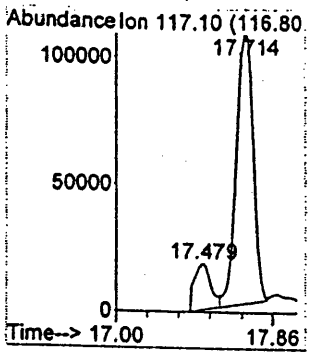
19

DHEA

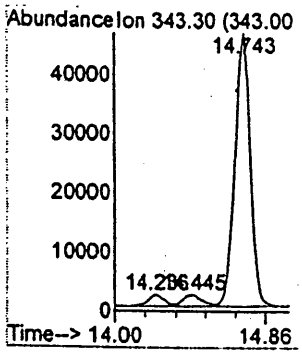




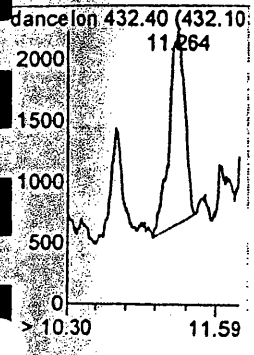
Prégnanediol



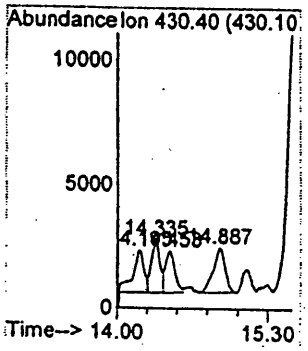
11Ketoetiocholanolone



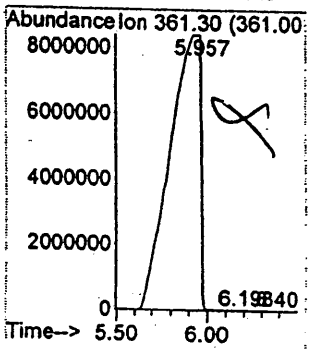
Androstandione



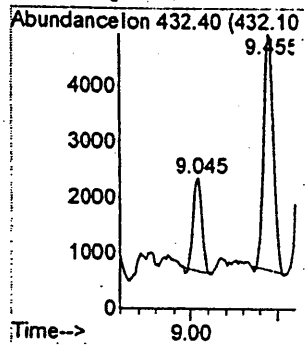
4 Androstenedione



Inhib derivation



3a-5 Cyclo



Sample 128/07 - 994276

PROCES-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM

BERLINGER

CONFIDENTIEL ! / CONFIDENTIAL !

2006
12807

Organisme demandant le contrôle / Organization requesting the test
- ☒ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☐ Fédération internationale

Type de contrôle / Type of test

Inopiné : Oui ☐ Non ☒

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Sexe / Sex: M ☒ F ☐

Fédération - Discipline / Sport: UCI - FF. CYCLISME

Nom de l'épreuve - Ville - CP / Event and place: TOUR DE FRANCE 1ère ET

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date: 14-07-2006

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate:

Nom et prénom du médecin / Name of doctor (en toutes lettres): OLIVIER DACHARY

Signature du Médecin / Signature of Doctor:

Réalisation du contrôle / Test completion

Date du prélèvement / Sampling date: 14-07-2006

Echantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code: BUR994276

Quantité totale d'urine, sang, ml / total amount of urine, blood, ml: 100 cc

Densité / Specific Gravity: 1.030

pH / pH: 5.5

Echantillon d'urine insuffisant / Insufficient urine sample

Médicaments / Drugs ☒ OUI ☐ NON

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used (éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Confirmation / Confirmation

Signature du Délégué Fédéral / Signature of Federal Delegate:

Signature du Médecin / Signature of Doctor:

Exemplaire destiné au Laboratoire National de Dépistage du dopage – 143, avenue Roger-Salengro – 92290 Châtenay-Malabry – Tél. : 01 46 60 28 (

USADA 0433

LNDD	ENREGISTREMENT FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS	Codification : E-Fiche_Labo-01 Version : Q Date : 18/05/2006 Page : 1 / 2

Numéro Labo: 28/07 Médecin BROCHARD Olivier Matériel: Berlinger Nbre 3

Sport Cyclisme (UCI) En compet (1)

Epreuve (F.I.) TDF 2006 étape n°12 Carcasonne

Date(s) de Prél.: 14/07/2006 Récept° Ech. : 14/07/2006 Récept° Doc. : 14/07/2006

Date de mise en tube : 15/07/2006 Date MT EPO : Date MT HES :

Observation Délai 48H

Ecarts: EA n°: Réclamat° N°:

N° :	994270	994276	995452									Operateur
Axe:	M	M	M									V20
Filtrat (F)												Operateur
volume	55	55	70									SL
pH-bande	5	5	6									FR
d-refract	1,027	1,026	1,035									23
ES06-Im	X	X	X									
ES02-Eph	X	X	X									
ES02-bbs	X	X	X									
ES04-H	X	X	X									
ES-Tg	2,15	2,10	0,98									
ES05-MS2	X	X	X									
ES03-cd	X	X	X									
ES03B-LCH	X	X	X									
ES08B-PS	X	X	X									
ES08-HES												
ES07-Epo												
ES01-HBO												
ESS02-TS												

Résultats :

Vérifié par : 18/07/06 Date : 04

A et B accouplés : le : par :

t B détruits: le : par :

LNDD	Enregistrement	Codification : E-Fiche_Labo-01 Version : Q Date 18/05/2006 Page : 2/2

FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS

CONCLUSIONS

ssai	Echantillon	RESULTATS ANORMAUX ET RESERVES	Date et Visa
S		Présence de pH d (+/-) Dépistage Conf	
S		Présence de pH d (+/-) Dépistage Conf	
S		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH d (+/-) Dépistage Conf	
S		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH d (+/-) Dépistage Conf	
		NB : les résultats de(s) l'essai(s) ES ne sont pas totalement exploitables en raison d'une inhibition de due à une interférence matricielle	

Si la place est insuffisante pour conclure, utiliser E-Fiche_Labo-01Bis en complément, dans ce cas, indiquer le nombre de feuilles utilisées :

RESULTATS NORMAUX	Date et Visa	RESULTATS NORMAUX	Date et Visa
Recherche négative pour les essais ES02C*, ES02, ES04 et ES05* Recherche négative pour les essais ES02C*, ES02, ES04 et ES05*	18/7/06	IMM RESULTAT DANS LES NO... POUR L'ESSAI ES06	18-07-06 VE
	Ecart :	EPO	Ecart :
Recherche négative pour les essais ES03 et ES03B (HA)	18/07/06	HES Recherche d'HEA négative pour l'essai ES08B*	18/7/06
	Ecart :		Ecart :

ion du (des) rapport(s) d'analyse : Visa

fication du(des) rapport(s) d'analyse par la LT et l'AQ ☒ Conv ☐ Spé Date et Visa : 18/07/06

Cet enregistrement est à archiver au département Logistique Technique

23

Data File Path D:\msd23\juil07\1507\
Data File Name **15h12.D**
Operator **M18p23/28**
Acq. Method File **MAN06B_B.M**
Sample Name **128/07 994276 H**
Misc Info
Vial Number **15**
Instrument Name **msd23**
Calibration Title **Calibration des stéroides endogènes**
Last Calibration Update **Sat Jul 15 10:18:12 2006**
Fichier recal utilise **15rcl01.D**

Date : 16/07/06
Opérateur : SF

CL [X] HC []

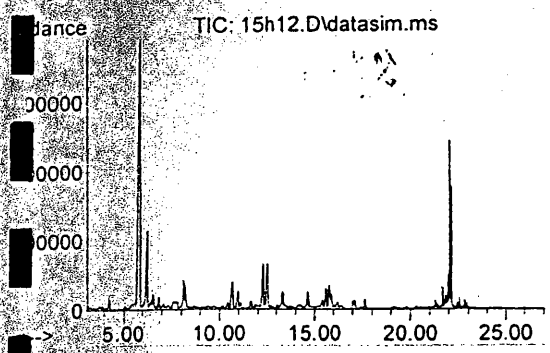
Résultats : Négatif [X]
A vérifier []

Remarques :
.....
..... 04

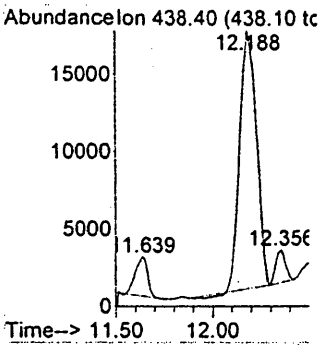
#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	17.02	301.3	Methyltestosterone	4,575,778	200	ng/ml
2)		12.19	438.4	Andro -D4 gluc	1,025,495	1020	ng/ml
3)		4.12	372.3	Salbutamol -D3	1,801,035	126	ng/ml
4)		12.26	434.4	Androsterone	16,257,387	1594	ng/ml
5)		12.50	434.4	Etiocholanolone	24,474,943	2041	ng/ml
6)		12.65	241.2	5a3a diol	547,120	85	ng/ml
7)		12.81	241.2	5b3a diol	2,086,476	333	ng/ml
8)		17.62	117.1	Pregnanediol	10,380,859	381	ng/ml
9)		14.64	434.4	DHT	563,078	74	ng/ml
10)		13.71	432.4	DHEA	315,031	28	ng/ml
11)		14.25	432.4	Epitestosterone	517,675	17	ng/ml
12)		15.13	432.4	Testosterone	770,376	21	ng/ml
13)		0.00	371.3	THC -M	0	0	ng/ml
14)		4.15	369.3	Salbutamol	362,658	-15	ng/ml
15)		15.88	522.5	11 bOH Etiocholanolone	8,079,265	1377	ng/ml
16)		21.28	548.5	THS	2,829,064	81	ng/ml
17)		22.06	636.6	THF	70,247,416	2733	ng/ml
18)		22.80	632.6	Cortisol	3,472,904	234	ng/ml
19)		14.64	343.3	11 Ketoetiocholanonlone	11,672,419	1080	ng/ml
20)		10.16	432.4	3a-5C	138,392		ng/ml

	[Surface]	[concentration]	Valeurs conventionnelles de rejet	
Ad4 glu / Si *100	22.4			**< 15.7**
T / ET	1.5		**Rapport**	**> 4**
Testosterone		21.5	**Concentration**	**> 200**
Epitestosterone		16.7	**Concentration**	**> 200**
Androsterone		1,594	**Concentration**	**> 10000**
Etiocholanolone		2,041	**Concentration**	**> 10000**
DHEA		28	**Concentration**	**> 200**
DHT		74	**Concentration**	**> 20**
Andro / Etio	0.7		**Rapport**	**> 3**
5a3a / 5b3a diol	0.3		**Rapport**	**> 2**
THC -M		0.0	**Concentration**	**> 15**
Salb/Salb D3 * 100	20	-15.2	**Concentration**	**> 75**
Cortisol / THS		2.9		
THF / THS		33.8		
11 bOH etio / THS		17.0	**Pour info (R&D)**	
THF		2733		
SI/3a5c	33.1			

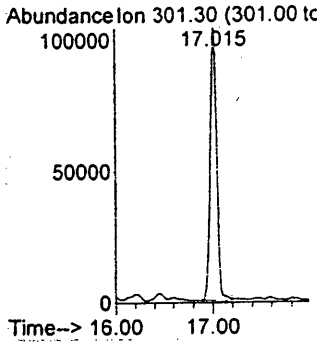
D:\MSD23\JUIL07\1507\15h12.D\da
cor: M18p23/28
Acquired: 15 Jul 06 8:23 pm
crument: msd23
thod File: MAN06B_B.M
ple Name: 128/07 994276 H
sc Info:
ial Number : 15



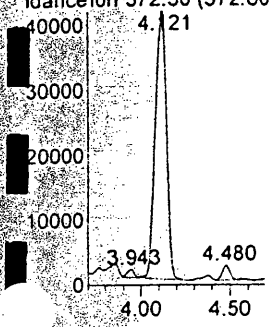
Andro -D4 gluc



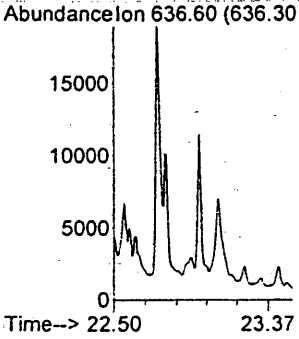
Methyltestosterone



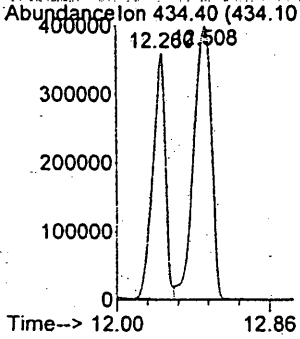
butamol -D3



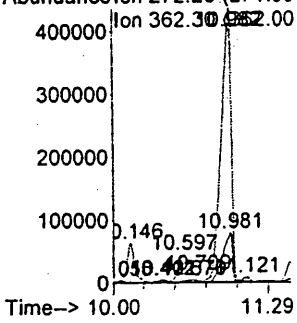
Cortisol D4



Andro-Etio di TMS

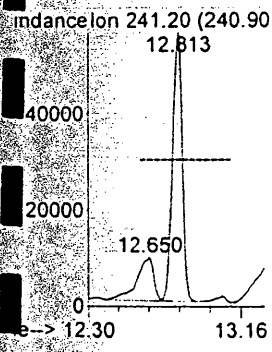


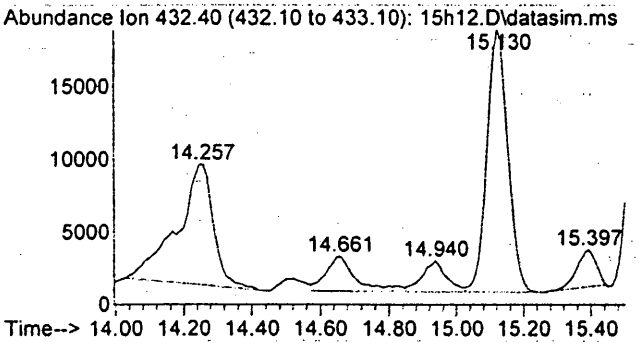
Andro-Etio mono TMS



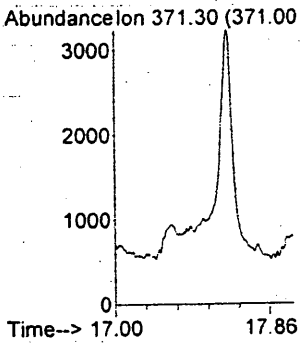
a-5b3a Diol

5b < trait : IRMS Epitestostérone - Testostérone

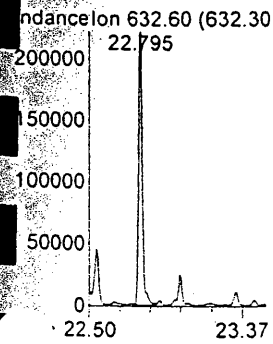




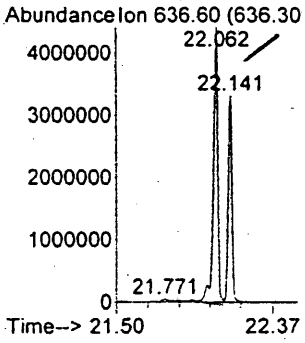
THC -M



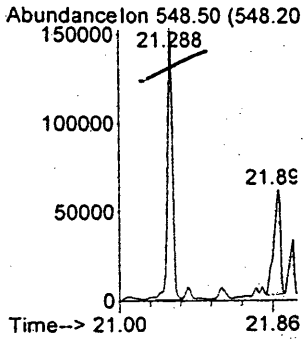
rtisol



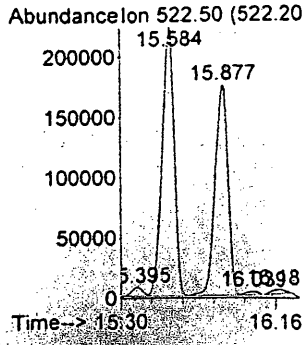
THF



THS



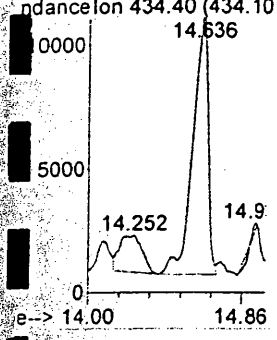
11 BOH A/E

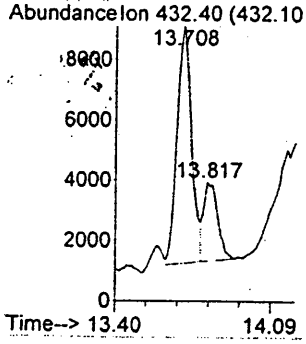


D:\MSD23\JUIL07\1507\15h12.D\da
M18p23/28
quired: 15 Jul 06 8:23 pm
ment: msd23
d File: MAN06B_B.M
le Name: 128/07 994276 H
c Info:
al Number : 15

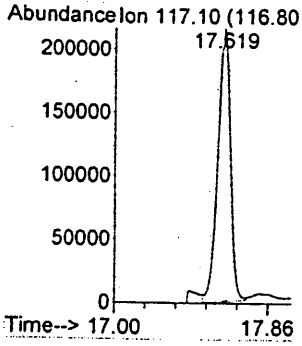
25

DHEA

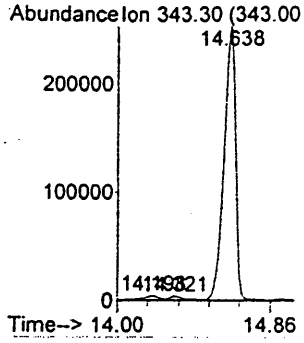




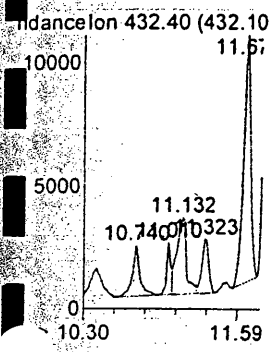
Prégnanediol



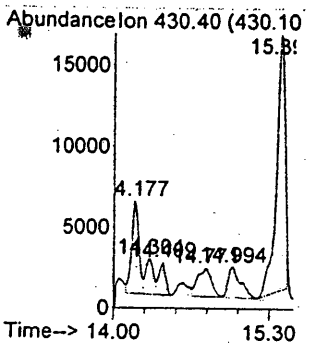
11Ketoetiocholanolone



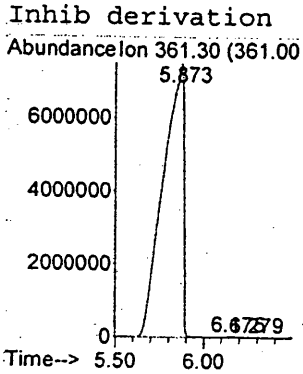
Androstandione



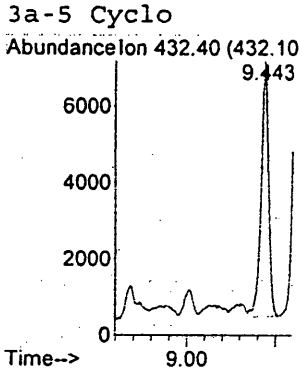
4 Androstenedione



Inhib derivation



3a-5 Cyclo



Sample 151/07 – 994075

PROCES-VERBAL DE CONTROLE ANTIDOPAGE / DOPING CONTROL FORM

ERLINGER

CONFIDENTIEL / CONFIDENTIAL

151.07 2006

Notification de contrôle et accès au réception / Notification at testing and subsequent arrival

Exemplaire A et B / Sample A and B

BUR994075

Echantillon d'urine insuffisant / Insufficient urine sample

Médicaments pris récemment / Drugs taken recently

Confirmation / Confirmation

Signature du Médecin / Signature of Doctor

Exemplaire destiné au Laboratoire National de Dépistage du dopage — 143, avenue Roger-Salengro — 92290 Châtenay-Malabry — Tél. : 01 46 60 28 6

USADA 0440

27

LNDD	ENREGISTREMENT FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS	Codification : E-Fiche_Labo-01 Version : Q Date : 18/05/2006 Page : 1 / 2

Numéro Labo: 151/07 **Médecin** TOLLENAERE Bruno **Matériel:** Berlinger **Nbre** 3

Sport Cyclisme (UCI) En compet (I)

Epreuve (F.I.) TDF 2006 étape n°15 a Alpes d'Huez

Date(s) de Prél.: 18/07/2006 **Récept° Ech. :** 19/07/2006 **Récept° Doc. :** 18/07/2006

Date de mise en tube : 19/07/06 **Date MT EPO :** 19-07-06 **Date MT HES :**

Observation Délai 48H EPO/HES

Ecarts: EA n°: **Réclamat° N° :**

N° :	994070	994075	994078								Operateur
Sexe:	M	M	M								V20
Filtrat (F)		F	F								Operateur
volume	90	65	70								44
pH-bande	5	5	5								44
d-refract	1,030	1,022	1,024								44
ES06-Im	α	α	α								
ES02-Eph	α	α	α								
ES02-bbs	α	α	α								
ES04-H	α	X	X								
Tg/ETg	0,1	1,3	1,0								
ES05-MS2	X	X	X								
ES03-cd	α	α	α								
ES03B-LCH	α	α	α								
ES08B-PS	α	α	α								
ES08-HES											
ES07-Epo	X	X	X								
ESS01:HBO											
ESS02-TS											

Résultats :

994070 et 994078 : Trenicrudone acétonide inférieure au seuil de confirmation
994075 : ~~méthylprednisolone~~ caféine (cdc) en attente surveillance AMA
994070 : Salbutamol (cdc) C = 119ng/ml (23) Ce 210706

Vérifié par : DP **Date :** 21/7/06

A et B accouplés : **le :** **par :**

A et B détruits: **le :** **par :**

FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS

CONCLUSIONS

Essai	Echantillon	RESULTATS ANORMAUX ET RESERVES	Date et Visa
ES		Présence de pH : d : (+/-.....) Dépistage : Conf :	
ES		Présence de pH : d : (+/-.....) Dépistage : Conf :	
ES 02 04	994070 (00)	Présence de Salbutamol à une concentration exprimée ~~supérieure au / proche~~ du seuil de 100 ng/mL : 119. ng/mL (+/- 20 %) nb : cause incertitude de mesure pH : 5.4 d : 1.029. (+/- 0.002) Dépistage : ES02, ES04 CG/SM Conf : EC18B CG/SM.	21/07/06 Seraphin
ES		Présence de à une concentration supérieure au / proche du seuil de ; (+/- %) pH : d : (+/-.....) Dépistage : Conf :	
ES ..		NB : les résultats de(s) l'essai(s) ES ne sont pas totalement exploitables en raison d'une inhibition de due à une interférence matricielle	

*Si la place est insuffisante pour conclure, utiliser **E-Fiche_Labo-01Bis** en complément ; dans ce cas, indiquer le nombre de feuilles utilisées :*

RESULTATS NORMAUX	Date et Visa	RESULTATS NORMAUX	Date et Visa
EC Recherche négative pour les essais ES02C*, ES02, ES04 et ES05*	21/7/6 Ecart :	IMM RÉSULTAT DANS LES NORMES POUR L'ESSAI ES06	200706 Ecart :
EC Recherche négative pour les essais ES03 et ES03B (HA)	20/07/06 Ecart :	EPO Absence d'EPO Ej et recombinants	21/07/06 Ecart :
		HES Recherche d'HEA négative pour l'essai ES08B*	21/7/6 Ecart :

Validation du (des) rapport(s) d'analyse : Visa :

Vérification du(des) rapport(s) d'analyse par la LT et l'AQ Conv Spé Date et Visa : 22/07/06

29

C:\MSD18\juillet06\2007\20h02.D

Data File Path C:\MSD18\juillet06\2007\
Data File Name 20h02.D
Operator m37p35
Acq. Method File MAN06B_B.M
Sample Name Q51/07 994075 H
Misc Info
Vial Number 5
Instrument Name GC MSD
Calibration Title Calibration des stéroides endogènes
Last Calibration Update Thu Jul 20 10:26:49 2006
Fichier recal utilise 20rcl01.D

Date : 2017/06
Opérateur : 37

C [] HC []

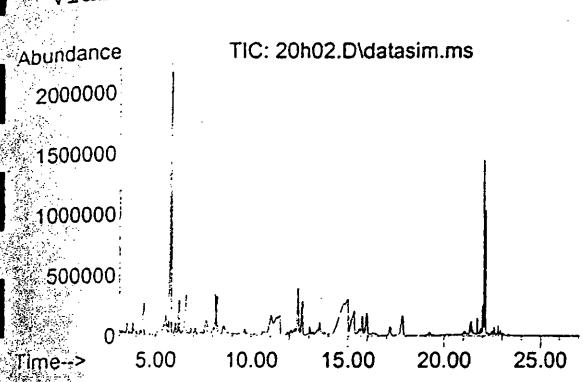
Résultats : Négatif [X]
A vérifier []

Remarques :
.....09

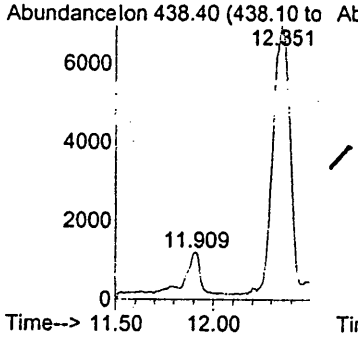
#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	17.17	301.3	Methyltestosterone	1,613,998	200	ng/ml
2)		12.35	438.4	Andro -D4 gluc	375,826	742	ng/ml
3)		4.19	372.3	Salbutamol -D3	1,139,948	199	ng/ml
4)		12.40	434.4	Androsterone	4,691,668	1017	ng/ml
5)		12.64	434.4	Etiocholanolone	3,789,961	823	ng/ml
6)		12.79	241.2	5a3a diol	66,585	27	ng/ml
7)		12.95	241.2	5b3a diol	181,645	71	ng/ml
8)		17.83	117.1	Pregnanediol	6,823,481	646	ng/ml
9)		0.00	434.4	DHT	0	0	ng/ml
10)		13.79	432.4	DHEA	165,805	34	ng/ml
11)		14.33	432.4	Epitestosterone	148,538	13	ng/ml
12)		15.33	432.4	Testosterone	274,727	22	ng/ml
13)		0.00	371.3	THC -M	0	0	ng/ml
14)		0.00	369.3	Salbutamol	0	-45	ng/ml
15)		15.74	522.5	11 bOH Etiocholanolone	2,575,586	847	ng/ml
16)		21.31	548.5	THS	1,110,810	95	ng/ml
17)		22.06	636.6	THF	19,610,174	2286	ng/ml
18)		22.81	632.6	Cortisol	1,009,858	227	ng/ml
19)		14.79	343.3	11 Ketoetiocholanonlone	926,120	228	ng/ml
20)		10.19	432.4	3a-5C	7,140		ng/ml

	[Surface]	[concentration]	Valeurs conventionnelles de rejet	
Ad4 glu / Si *100	23.3			< 19.1
T / ET	1.8		Rapport	> 4
Testosterone		22.3	Concentration	> 200
Epitestosterone		13.5	Concentration	> 200
Androsterone		1,017	Concentration	> 10000
Etiocholanolone		823	Concentration	> 10000
DHEA		34	Concentration	> 200
DHT		0	Concentration	> 20
Andro / Etio	1.2		Rapport	> 3
5a3a / 5b3a diol	0.4		Rapport	> 2
THC -M		0.0	Concentration	> 15
Salb/Salb D3 * 100	0	-45.0	Concentration	> 75
Cortisol / THS		2.4		
THF / THS		24.1		
11 bOH etio / THS		8.9	Pour info (R&D)	
THF		2286		
SI/3a5c	226.1			

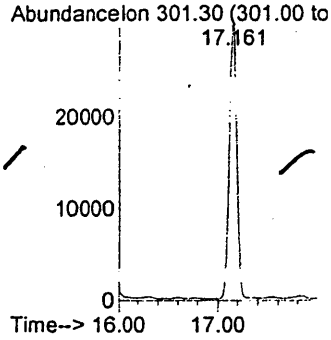
Data File : C:\MSD18\JUILLET06\2007\20h02.D\da
Operator: m37p35
Date Acquired: 20 Jul 06 12:22 pm
Instrument: GC MSD
Method File: MAN06B_B.M
Sample Name: 151/07 994075 H
Misc Info:
Vial Number : 5



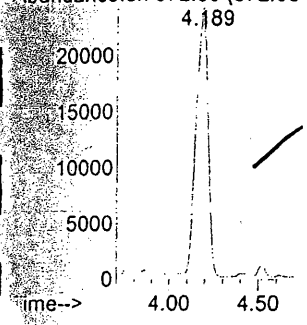
Andro -D4 gluc



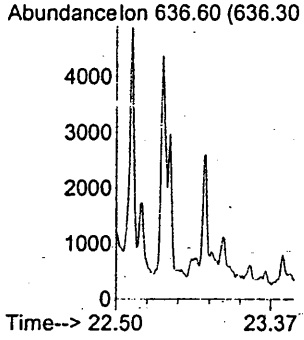
Methyltestosterone



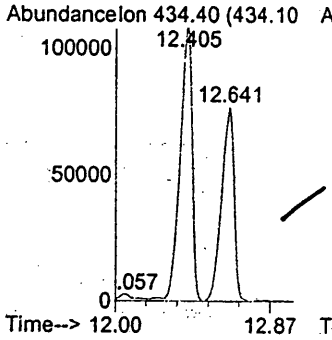
Salbutamol -D3



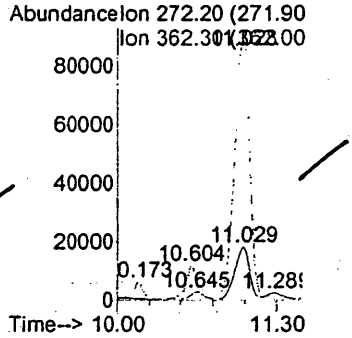
Cortisol D4



Andro-Etio di TMS

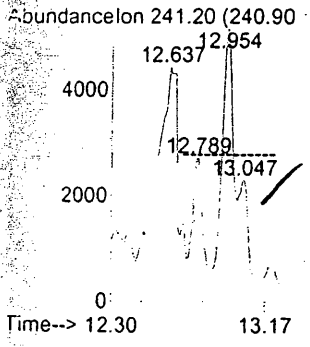


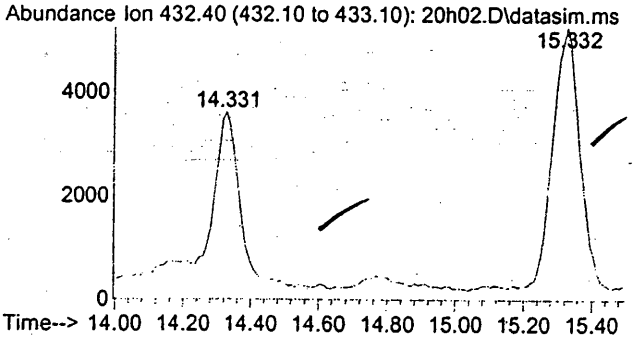
Andro-Etio mono TMS



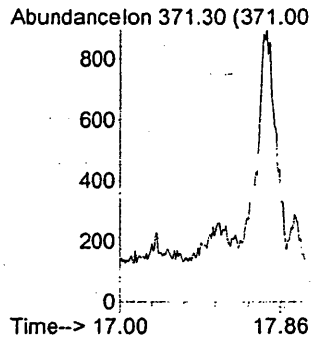
a3a-5b3a Diol

si 5b < trait : IRMS Epitestostérone - Testostérone

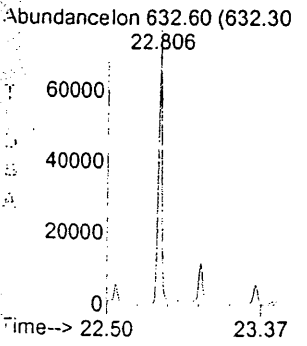




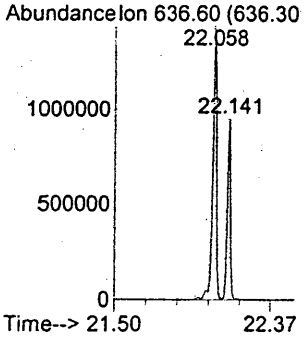
THC -M



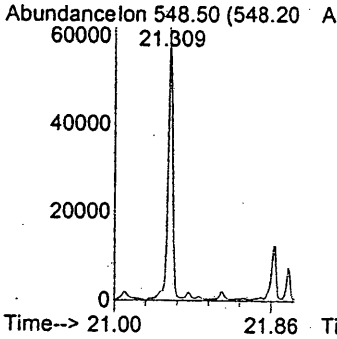
Cortisol



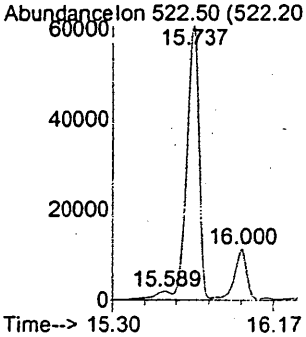
THF



THS

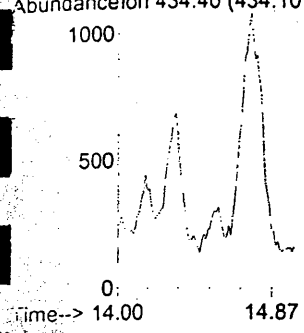


11 BOH A/E

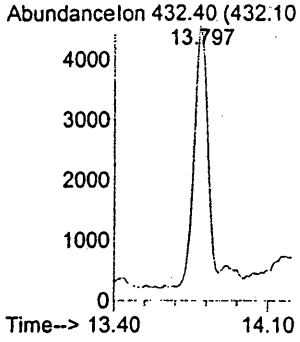


C:\MSD18\JUILLET06\2007\20h02.D\da
...or: m37p35
...Acquired: 20 Jul 06 12:22 pm
...rument: GC MSD
...thod File: MAN06B_B.M
...ample Name: 151/07 994075 H
Misc Info:
Vial Number : 5

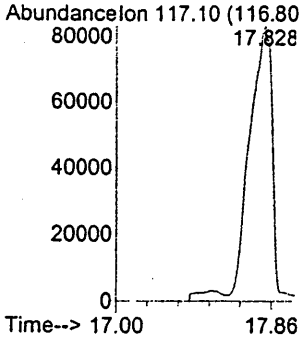
DHT



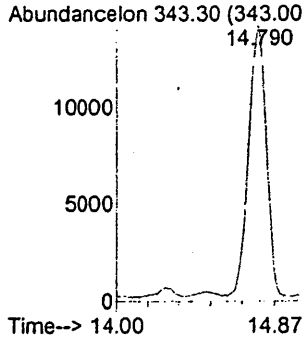
DHEA



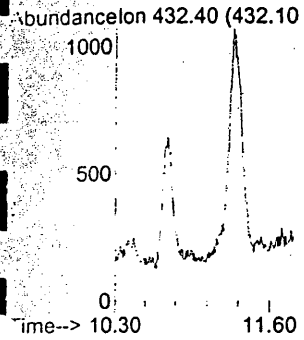
Prégnanediol



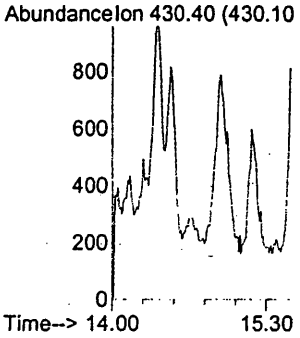
11Ketoetiocholanolone



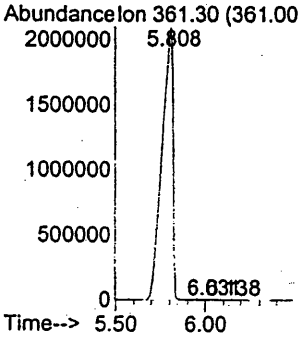
5b Androstandione



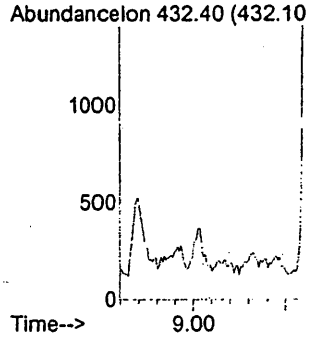
4 Androstenedione



Inhib derivation



3a-5 Cyclo



Sample 178/07 – 995474

PROCES-VERBAL DE CONTROLE ANTIDOPAGE / DOPING CONTROL FORM

CONFIDENTIEL / CONFIDENTIAL

17.07.2006

Organisme demandant le contrôle / Organization requesting the test

☐ Ministère chargé des Sports

☐ Direction régionale de la Jeunesse et des Sports

☐ Fédération nationale

☒ Fédération internationale

Type de contrôle / Type of test

Inopiné / Out ☐ Non ☒

Sexe / Sex M ☒ F ☐

Compétition: Internationale ☒ Nationale ☐ Régionale ☐

Hors compétition: En club ☐ Stage hors club ☐ Cabinet médical ☒ Suivi ☐

Fédération - Discipline / Sport

Vel - Etc Cyclisme

Nom de l'épreuve - Ville - CP / Event and place

Tour de France - 17ème Etape

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date 20/07/2006

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate

Nom et prénom du médecin / Name of doctor (en toutes lettres)

Signature du Médecin / Signature of Doctor

Réalisation du contrôle / Test completion

Date du prélèvement / Sampling date 20/07/2006

Echantillons A et B / Samples A and B

Code Flacon (et container) n° / bottle Code

BUR995474

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml 125 ml

Densité / Density

Echantillon d'urine insuffisant / Insufficient urine sample

Médicaments / Drugs OUI ☐ NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used (éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Confirmation / Confirmation

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du Médecin / Signature of Doctor

Exemplaire destiné au Laboratoire National de Dépistage du dopage – 143, avenue Roger-Salengro – 92290 Châtenay-Malabry – Tél. : 01 46 60 2

USADA 0447

LNDD	ENREGISTREMENT FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS	Codification : E-Fiche_Labo-01 Version : Q Date : 18/05/2006 Page : 1 / 2

Numéro Labo: 178/07 Médecin BORDABERRY Gérard Matériel: Berlinger Nbre 3

Sport Cyclisme (UCI) En compet / H

Epreuve (F.I.) TDF 2006 étape n°17 à Morzine

Date(s) de Prél.: 20/07/2006 Récept° Ech. : 20/07/2006 Récept° Doc. : 20/07/2006

Date de mise en tube : 21/07/06 - 07 Date MT EPO : [illegible] Date MT HES : [illegible]

Observation Délai 48H EPO/HES

Ecarts: EA n°: Réclamat° N° :

N° :	994178	994179	995474									Operateur
Sexe:	M	M	M									V20
Filtrat (F)												Operateur
volume	95	85	65									44
pH-bande	5	7	5									44
d-réfract	1,030	1,018	1026									44
ES06-Im	X	X	X									
ES02-Eph	X	X	X									
ES02-bbs	X	X	X									
ES04-H	X	X	X									
ES05-MS2	X	X	X									
ES03-cd	X	X	X									
ES03B-LCH	X	X	X									
ES08B-PS	X	X	X									
ES09-HES	X	X	X									
ES07-Epo	X	X	X									
ES01-HBO												
ES02-TS												

Résultats :

995474 : TIE (dc) P=11 @ le 21/07/06 à 2
présence de 2 métabolites de dérivation

994178 } Testostérone actuelle inférieure au seuil de confirmation ne pas confirmer A
994179 : Cafeine (dc) en optique surveillance AMA.
994179 : Morphine < seuil

Vérifié par : Date : 27/7/6

A et B accouplés : le : 28/07/06 à 15h45 par : V8

A et B détruits: le : par :

LNDD	Enregistrement	Codification : E-Fiche_Labo-01 Version : Q Date 18/05/2006 Page : 2/2

FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS

CONCLUSIONS

Essai	Echantillon	RESULTATS ANORMAUX ET RESERVES	Date et Visa
ES....		Présence de pH d (+/-) Dépistage Conf	
S.....		Présence de pH d (+/-) Dépistage Conf	
S.....		Présence de à une concentration supérieure au / proche du seuil de(+/- %) pH d (+/-) Dépistage Conf	
S.....		Présence de à une concentration supérieure au / proche du seuil de(+/- %) pH d (+/-) Dépistage Conf	
ES04 ES05	995475	NB : les résultats de(s) l'essai(s) ES04 et ES05 ne sont pas totalement exploitables en raison d'une inhibition de la dilution due à une interférence matricielle	23/7/06
ES04	995476	Théophé supérieur au seuil de la CI (+/-30%) Concestéophée et corrigée : 4,5 mg/ml (+/-20%) Concepi théophé et corrigée : 3,9 mg/ml (+/-30%) pH=5,2 d=1,03 1,025 (+/-0,001) screening C6/SM (+/-30%) conf C6/SM CEC70	20/07/06 Conforme

Si la place est insuffisante pour conclure, utiliser E-Fiche_Labo-01Bis en complément ; dans ce cas, indiquer le nombre de feuilles utilisées : 1

RESULTATS NORMAUX	Date et Visa	RESULTATS NORMAUX	Date et Visa
Pour 995476 : ES02, ES02C, ES05 : négatif Recherche négative pour les essais ES02, ES04 et ES05*	23/7/06	IMM CLT dans les normes pour l'essai ES06	20/07/06 Ecart :
	Ecart :	EPO 994178 : négatif autres : absence d'EPO recombinante ES01	23/07/06 Ecart :
Recherche négative pour les essais ES03 et ES03B (HA)	22/7/06 Ecart :	HES Recherche d'HEA négative pour l'essai ES08B*	23/7/06 Ecart :

Validation du (des) rapport(s) d'analyse : Visa :

Certification du(des) rapport(s) d'analyse par la LT et l'AQ ☒ Conv ☒ Spé Date et Visa : AM 25/07/06

Cet enregistrement est à archiver au département Logistique Technique

LNDD	ENREGISTREMENT	Codification : E-Fiche-labo-01BIS Version : A Date : 01/07/2004 1/1
FICHE COMPLEMENTAIRE POUR LA CONCLUSION DES RESULTATS ANALYTIQUES		

Numéro de la feuille: 1

Numéro de Labo: 178/07

Analyses concernées:

☐ analyses Conventionnelles Chimie GC ☐ analyses Conventionnelles Chimie LC
☐ analyses Conventionnelles Immunochimie
☒ analyses Spécialisées Chimie GC: ☐ HES ☒ 12C/13C
☐ analyses Spécialisées Biologie: EPO
☐ analyses Spécialisées Biologie (et Chimie): HBOCs

CONCLUSION (à dater et signer par le Responsable technique):

995474 : l'analyse de l'échantillon par spectro de masse de rapport isotopiq (CC31) indique une origine exogène des métabolites de la testostérone, cohérente avec une prise de testo ou de l'un de ses précurseurs.
L'origine exogène des métabolites de la testostérone a été objectivée sur la base d'un appauvrissement isotopique de 3,99‰ et 6,14‰, respectivement pour les métabolites androstérone et 5α androstanediol.
Seuil de positivité de l'AFLA : appauvrissement isotopiq > 3‰ (± 0,8‰ interne au laboratoire).

pH = 5,2 d = 1,025

le 25/07/06

36

C:\MSD18\juillet06\2107\21h08.D

Data File Path C:\MSD18\juillet06\2107\
Data File Name 21h08.D
Operator m37p35
Acq. Method File MAN06B_B.M
Sample Name 178/07 995474 H
Misc Info
Vial Number 11
Instrument Name GC MSD
Calibration Title Calibration des stéroides endogènes
Last Calibration Update Fri Jul 21 13:26:58 2006
Fichier recal utilise 21rcl03.D

Date : 22107106
Opérateur : 18

C [] HC []

Résultats : Négatif []
A vérifier [X]

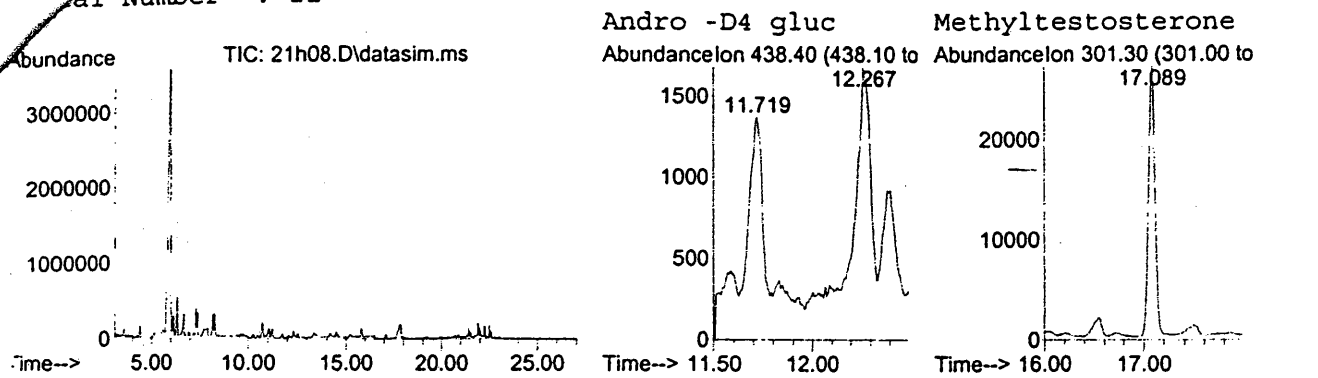
Remarques : TIE
Inhibiteur de dérivation

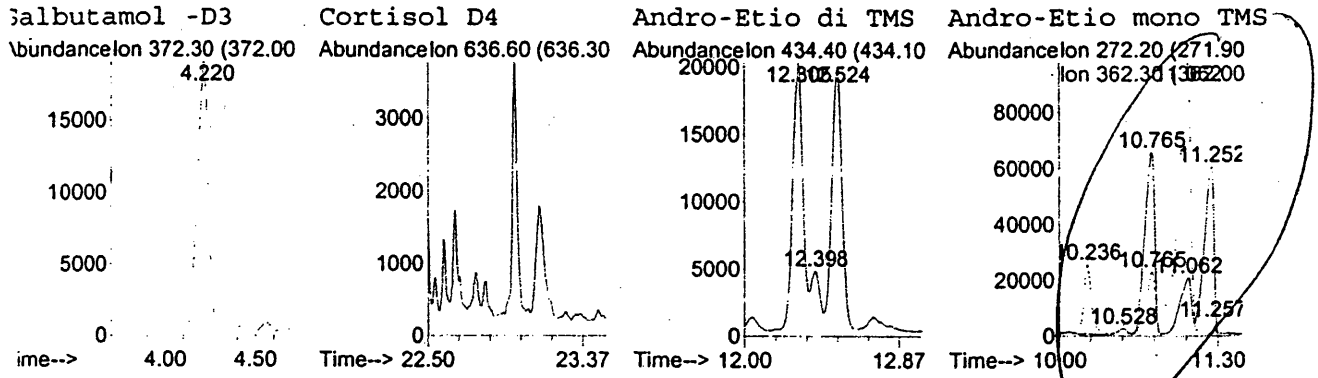
#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	17.09	301.3	Methyltestosterone	1,209,250	200	ng/ml
2)		12.27	438.4	Andro -D4 gluc	62,604	163	ng/ml
3)		4.22	372.3	Salbutamol -D3	814,633	226	ng/ml
4)		12.30	434.4	Androsterone	846,467	230	ng/ml
5)		12.52	434.4	Etiocholanolone	813,037	215	ng/ml
6)		12.70	241.2	5a3a diol	197,002	96	ng/ml
7)		12.88	241.2	5b3a diol	439,619	224	ng/ml
8)		17.85	117.1	Pregnanediol	10,142,344	1316	ng/ml
9)		0.00	434.4	DHT	0	0	ng/ml
10)		0.00	432.4	DHEA	0	0	ng/ml
11)		14.30	432.4	Epitestosterone	116,467	14	ng/ml
12)		15.20	432.4	Testosterone	575,745	61	ng/ml
13)		0.00	371.3	THC -M	0	0	ng/ml
14)		0.00	369.3	Salbutamol	0	-45	ng/ml
15)		0.00	522.5	11 bOH Etiocholanolone	0	0	ng/ml
16)		0.00	548.5	THS	0	0	ng/ml
17)		0.00	636.6	THF	0	0	ng/ml
18)		0.00	632.6	Cortisol	0	0	ng/ml
19)		14.68	343.3	11 Ketoetiocholanonlone	183,640	65	ng/ml
20)		10.25	432.4	3a-5C	23,035		ng/ml

	[Surface]	[concentration]	Valeurs conventionnelles de rejet	
Ad4 glu / Si *100	5.2			< 19.1
T / ET	4.9		Rapport	> 4
Testosterone		60.6	Concentration	> 200
Epitestosterone		13.7	Concentration	> 200
Androsterone		230	Concentration	> 10000
Etiocholanolone		215	Concentration	> 10000
DHEA		0	Concentration	> 200
DHT		0	Concentration	> 20
Andro / Etio	1.0		Rapport	> 3
5a3a / 5b3a diol	0.4		Rapport	> 2
THC -M		0.0	Concentration	> 15
Salb/Salb D3 * 100	0	-45.0	Concentration	> 75
Cortisol / THS		#DIV/0!		
THF / THS		#DIV/0!		
11 bOH etio / THS		#DIV/0!	Pour info (R&D)	
THF		0		
SI/3a5c	52.5			

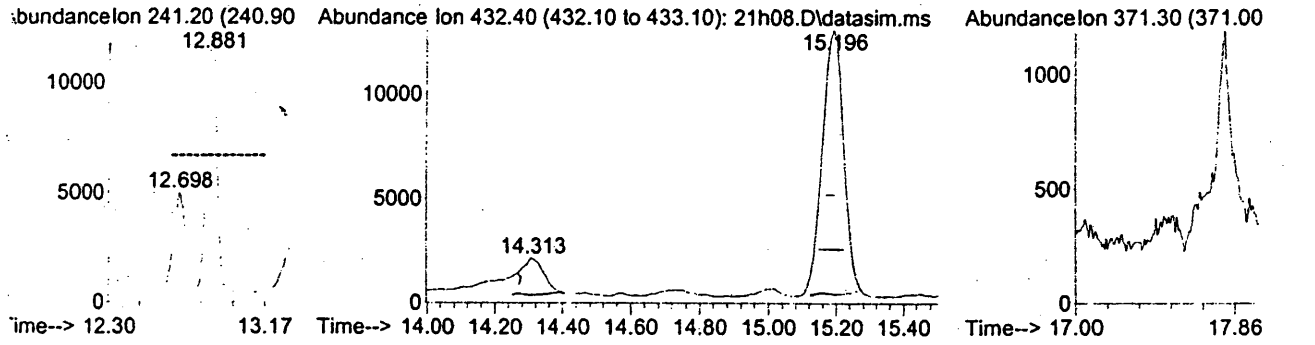
C:\MSD18\JUILLET06\2107\21h08.D\da
m37p35
ed: 21 Jul 06 7:36 pm
t: GC MSD
File: MAN06B_B.M
e Name: 178/07 995474 H
e Info:
al Number : 11

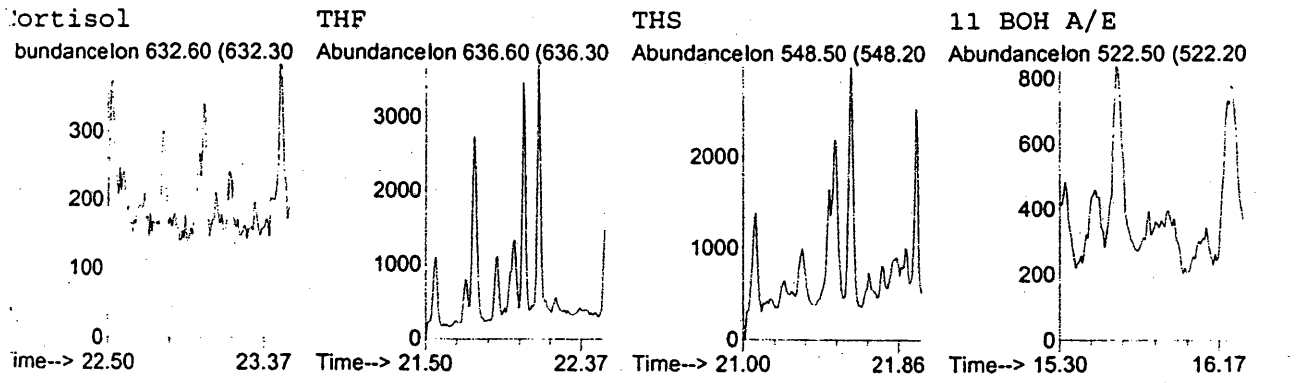
37





a3a-5b3a Diol
i 5b < trait : IRMS Epitestostérone - Testostérone





MSD18\JUILLET06\2107\21h08.D\da
37p35
21 Jul 06 7:36 pm
GC MSD
File: MAN06B_B.M
Name: 178/07 995474 H
Sc Info:
Vial Number : 11

30

DHT
Abundance Ion 434.40 (434.10
600
400
200
0
Time--> 14.00 14.87

DHEA
Abundance Ion 432.40 (432.10
1000
500
0
Time--> 13.40 14.10

Prégnanediol
Abundance Ion 117.10 (116.80
80000
60000
40000
20000
0
17.85
17.749
Time--> 17.00 17.86

11Ketoetiocholanolone
Abundance Ion 343.30 (343.00
4000
3000
2000
1000
0
14.685
Time--> 14.00 14.87

b Androstandione
bundance Ion 432.40 (432.10
1500
1000
500
0
11.230
Time--> 10.30 11.60

4 Androstenedione
Abundance Ion 430.40 (430.10
800
600
400
200
0
Time--> 14.00 15.30

Inhib derivation
Abundance Ion 361.30 (361.00
3000000
2000000
1000000
0
6.002
6.162637
Time--> 5.50 6.00

3a-5 Cyclo
Abundance Ion 432.40 (432.10
2000
1500
1000
500
0
9.112
Time--> 9.00

D:\msd19\juil07\2507\17807474.D

39

Data File Path D:\msd19\juil07\2507\
Data File Name **17807474.D**
Operator **M45P35**
Acq. Method File **MAN06B_B.M**
Sample Name **178/07 995474 H**
Misc Info
Vial Number 2
Instrument Name **MSD 19**
Calibration Title **Calibration des stéroides endogènes**
Last Calibration Update **Tue Jul 25 15:15:32 2006**
Fichier recal utilise **25rcl01.D**

Date : 25/07/06
Opérateur : ...10...

C [] HC []

Résultats : Négatif []
A vérifier []

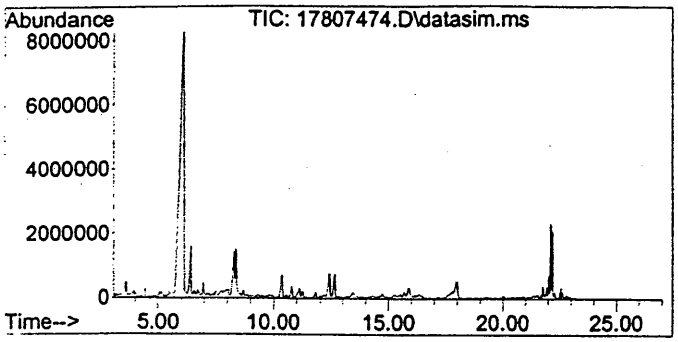
Remarques : Vial de conf réinjecté
Is screening

#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	17.14	301.3	Methyltestosterone	1,731,969	100	ng/ml
2)		12.38	438.4	Andro -D4 gluc	104,768	170	ng/ml
3)		0.00	372.3	Salbutamol -D3	0	0	ng/ml
4)		12.41	434.4	Androsterone	9,973,015	1658	ng/ml
5)		12.64	434.4	Etiocholanolone	11,119,071	1639	ng/ml
6)		12.78	241.2	5a3a diol	524,770	97	ng/ml
7)		12.94	241.2	5b3a diol	1,240,694	227	ng/ml
8)		17.79	117.1	Pregnanediol	19,393,646	487	ng/ml
9)		0.00	434.4	DHT	0	0	ng/ml
10)		13.81	432.4	DHEA	151,077	21	ng/ml
11)		14.35	432.4	Epitestosterone	214,591	11	ng/ml
12)		15.23	432.4	Testosterone	1,093,773	50	ng/ml
13)		0.00	371.3	THC -M	0	0	ng/ml
14)		0.00	369.3	Salbutamol	0	#DIV/0!	ng/ml
15)		15.98	522.5	11 bOH Etiocholanolone	554,334	130	ng/ml
16)		21.35	548.5	THS	1,376,986	88	ng/ml
17)		22.11	636.6	THF	32,301,427	2915	ng/ml
18)		22.85	632.6	Cortisol	1,008,236	143	ng/ml
19)		14.73	343.3	11 Ketoetiocholanonlone	2,541,956	309	ng/ml
20)		0.00	241.2	3a-5C	0		ng/ml

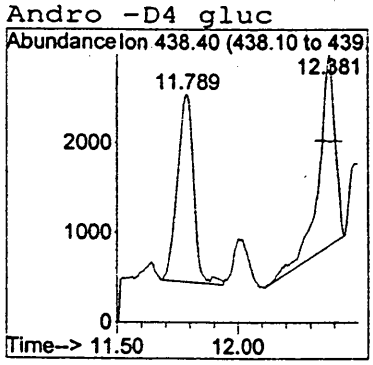
	[Surface]	[concentration]	Valeurs conventionnelles de rejet	
Ad4 glu / Si *100	6.0			**< 12.9**
T / ET	5.1		**Rapport**	**> 4**
Testosterone		49.7	**Concentration**	**> 200**
Epitesto		11.1	**Concentration**	**> 200**
Androsterone		1,658	**Concentration**	**> 10000**
Etiocholanolone		1,639	**Concentration**	**> 10000**
DHEA		20.6	**Concentration**	**> 200**
DHT		0.0	**Concentration**	**> 20**
Andro / Etio	0.9		**Rapport**	**> 3**
5a3a / 5b3a diol	0.4		**Rapport**	**> 2**
THC -M		0.0	**Concentration**	**> 15**
Salb/Salb D3 * 100	#DIV/0!	#DIV/0!	**Concentration**	**> 75**
Cortisol / THS		1.6		
THF / THS		33.1		
11 bOH etio / THS		1.5	**Pour info (R&D)**	
THF		2915		
SI/3a5c	#DIV/0!			

File: D:\MSD19\JUIL07\2507\17807474.D\da
Operator: M45P35
Date Acquired: 25 Jul 06 10:42 am
Instrument: MSD 19
Method File: MAN06B_B.M
Sample Name: 178/07 995474 H
Misc Info:
Vial Number : 2

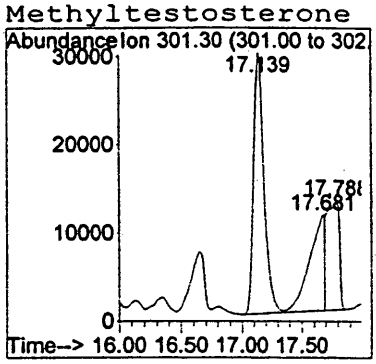
UP



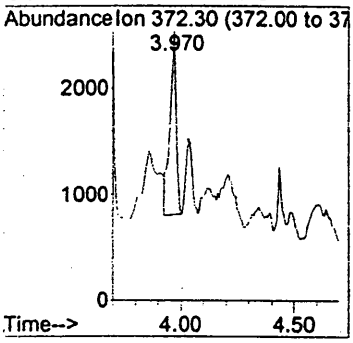
Andro -D4 gluc



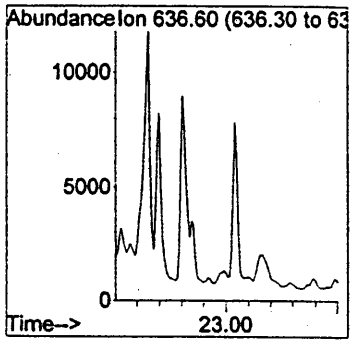
Methyltestosterone



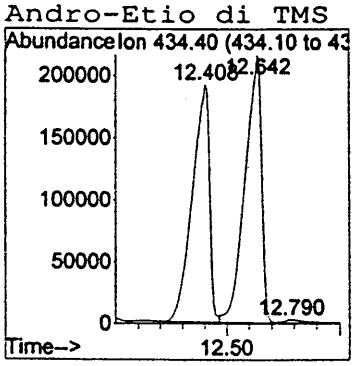
Salbutamol -D3



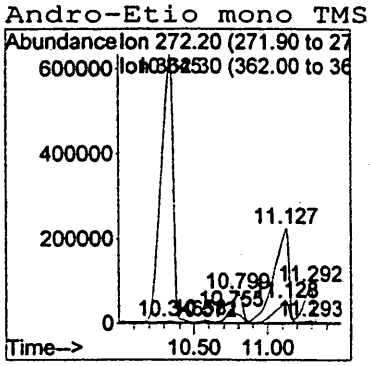
Cortisol D4



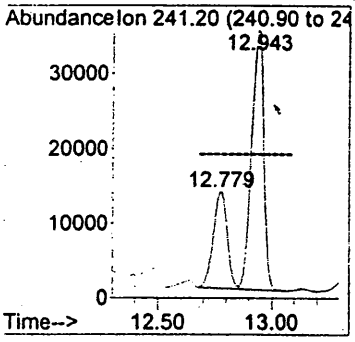
Andro-Etio di TMS

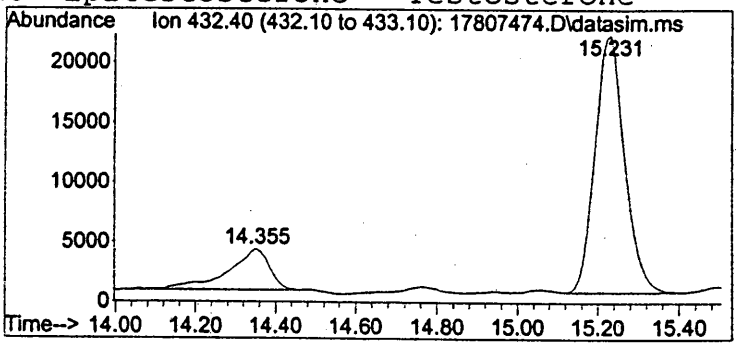


Andro-Etio mono TMS

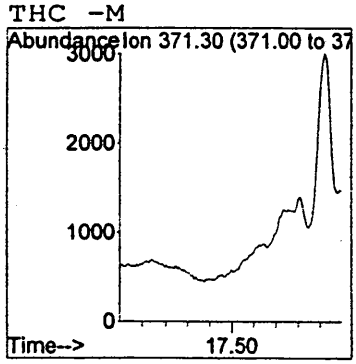


5a3a-5b3a Diol
si 5b < trait : IRMS Epitestostérone - Testostérone

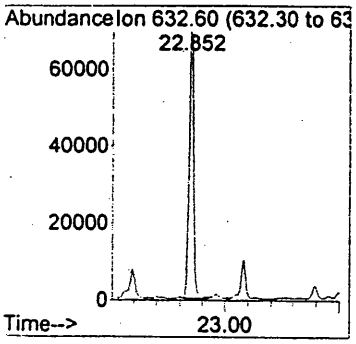




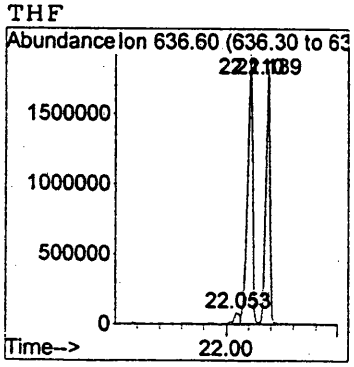
THC -M



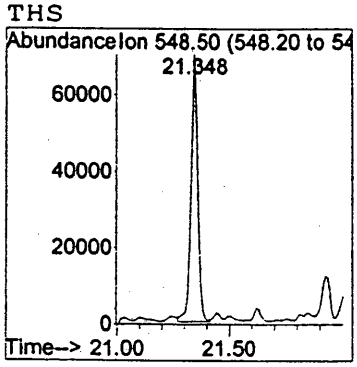
Cortisol



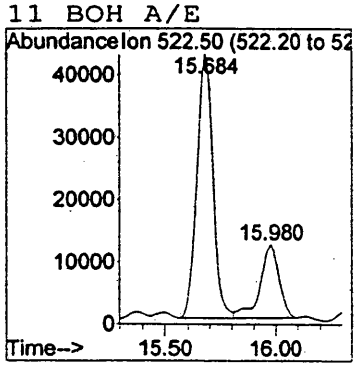
THF



THS



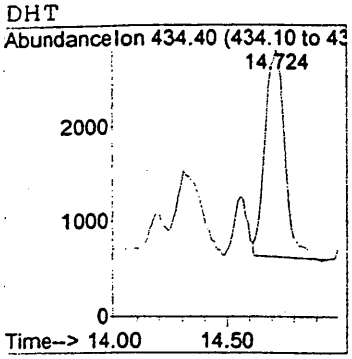
11 BOH A/E



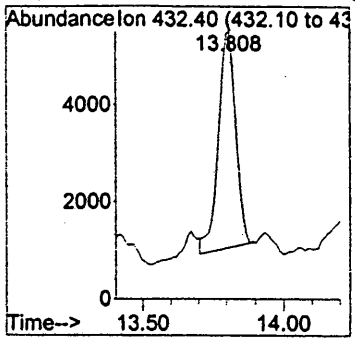
e: D:\MSD19\JUIL07\2507\17807474.D\da
erator: M45P35
ate Acquired: 25 Jul 06 10:42 am
Instrument: MSD 19
Method File: MAN06B_B.M
Sample Name: 178/07 995474 H
Misc Info:
Vial Number : 2

u1

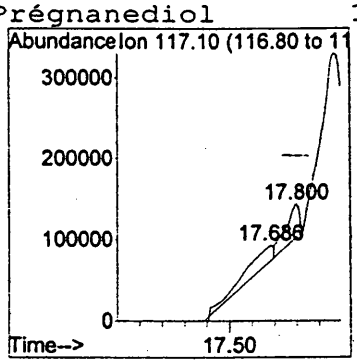
DHT



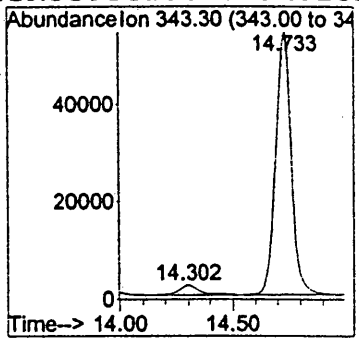
DHEA



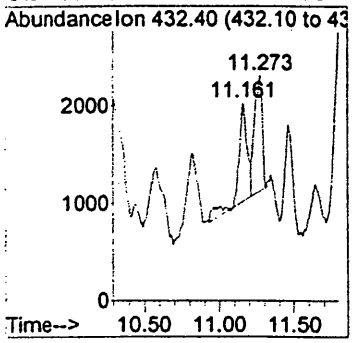
Prégnanediol



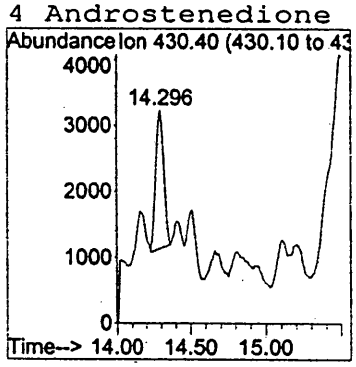
11Ketoetiocholanolone



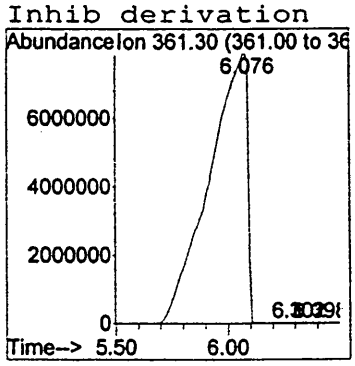
5b Androstandione



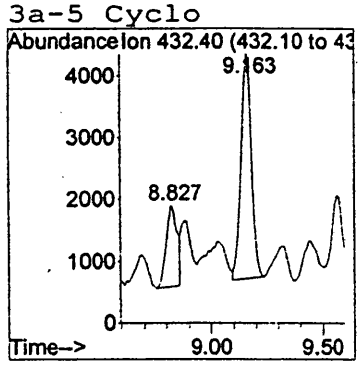
4 Androstenedione



Inhib derivation



3a-5 Cyclo



Sample 188/07 – 994080

42

PROCES-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL / CONFIDENTIAL

RERLINGE

2006
18.07

Organisme demandant le contrôle / Organization requesting test
- Ministère chargé des Sports
- Direction régionale de la Jeunesse et des Sports
- Fédération nationale
- Fédération internationale ☒

Type de contrôle / Type of test
Inopiné / Surprise : Oui ☐ Non ☒

Sexe / Sex : M ☒ F ☐

Compétition
Internationale ☒
Nationale ☐
Régionale ☐

Hors compétition
En club ☐
Stage hors club ☐
Cabinet médical ☐
Suivi ☐

Fédération - Discipline / Sport
VOL - ATC CYCLISME

Nom de l'épreuve - Ville - CP / Event and place
TOUR DE FRANCE - VIRE

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 22/07/2006

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate
MERIVIGETA GUY JEAN

Nom et prénom du médecin / Name of doctor (en toutes lettres)
BARDAGANY GERARD

Signature du Médecin / Signature of Doctor

Réalisation du contrôle / Test completion

Date du prélèvement / Sampling date : 22/07/2006

Echantillons A et B / Samples A and B
Code flacon (et container) n° / bottle Code
BUR994080

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 ml

Densité / Specific Gravity : 1,020

pH / pH : 5

Echantillon d'urine insuffisant / Insufficient urine sample

Médicaments / Drugs : OUI ☒ NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used
(éventuellement nom du médecin prescripteur)

Commentaires sur la procédure / Comments on procedure

Confirmation / Confirmation

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du Médecin / Signature of Doctor

Exemplaire destiné au laboratoire National de Dépistage du dopage – 143, avenue Roger-Salengro – 92290 Châtenay-Malabry – Tél. : 01 46

USADA 0458

LNDD	ENREGISTREMENT FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS	Codification : E-Fiche_Labo-01 Version : Q Date : 18/05/2006 Page : 1 / 2

Numéro Labo: 188/07 | Médecin: BORDABERRY Gérard | Matériel: Berlinger | Nbre: 3

Sport: Cyclisme (UCI) | En compet (1)

Epreuve (F.I.): TDF 2006 étape n°19 à Monteau les mines

Date(s) de Prél.: 22/07/2006 | Récept° Ech. : 22/07/2006 | Récept° Doc. : 22/07/2006

Date de mise en tube : 23/07/06 | Date MT EPO : 23/07/2006 | Date MT HES :

Observation: Délai 48H EPO/HES

Ecarts: EA n° : 103 | Réclamat° N° :

	994080	994187	995476									Operateur
Sexe:	M	M	M									V20
Filtrat (F)	F											Operateur
volume	60	85	75									44
pH-bande	5	5	5									44
d-refract	1,023	1,028	1,008									44
ES08-Int	X	X	X									
Eph	X	X	X									
2-bbs	X	X	X									
ES01-H	X	X	X									
1g/Etg	2,5	1,1	1,1									
ES05-MS2	X	X	X									
ES03-cd	X	X	X									
ES03B-LCH	X	X	X									
ES08B-PS	X	X	X									
ES08-HES												
Epo	X	X	X									
ESS01-HBO												
ESS02-TS												

Résultats :

994187 Terbutaline / codéine en attente surveillance rmn

994187 Budesonide en cC seuil d'envoi en c-f 15

Vérifié par : 09 Date : 26/4/06

B accouplés : le : par :

A et B détruits: le : par :

LNDD	Enregistrement	Codification : E-Fiche_Labo-01 Version : Q Date 18/05/2006 Page : 2/2

FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS

CONCLUSIONS

essai	Echantillon	RESULTATS ANORMAUX ET RESERVES	Date et Visa
ES...		Présence de pH : d : (+/-) Dépistage : Conf :	
S.....		Présence de pH : d : (+/-) Dépistage : Conf :	
ES.....		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH : d : (+/-) Dépistage : Conf :	
ES.....		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH : d : (+/-) Dépistage : Conf :	
ES 04 05	9316010	NB : les résultats de(s) l'essai(s) ES04 et ES05 ne sont pas totalement exploitables en raison d'une inhibition de la dérivation due à une interférence matricielle	26/7/16

Si la place est insuffisante pour conclure, utiliser E-Fiche_Labo-01Bis en complément, dans ce cas, indiquer le nombre de feuilles utilisées :

RESULTATS NORMAUX	Date et Visa	RESULTATS NORMAUX	Date et Visa
Recherche négative pour les essais ES02C*, ES02, ES04 et ES05*	26/7/16	IMM : RESULTAT DANS LES NORMES POUR L'ESSAI ES06	240706
	Ecart : 103 RAS	EPO : Absence d'EPO recombinants pour l'essai ES07	250706
Recherche négative pour les essais ES03 et ES03B (HA)	25/07/16		Ecart :
	Ecart :	HES : Recherche d'HEA négative pour l'essai ES08B*	26/7/16
			Ecart :

Edition du (des) rapport(s) d'analyse : Visa :

Vérification du(des) rapport(s) d'analyse par la LT et l'AQ : X Conv Date et Visa : 4H 26/7/16

D:\MSD19\JUIL07\2407\24h01.D

Data File Path D:\MSD19\JUIL07\2407\
Data File Name **24h01.D**
Operator **m45p35**
Acq. Method File **MAN06B_B.M**
Sample Name **R 188/07 994080 H**
Misc Info
Vial Number **4**
Instrument Name **MSD 19**
Calibration Title **Calibration des stéroides endogènes**
Last Calibration Update **Mon Jul 24 11:26:39 2006**
Fichier recal utilise **24rcl01.D**

Date : 25/04/16
Opérateur : G.F.

C [] HC []

Résultats : Négatif []
A vérifier []

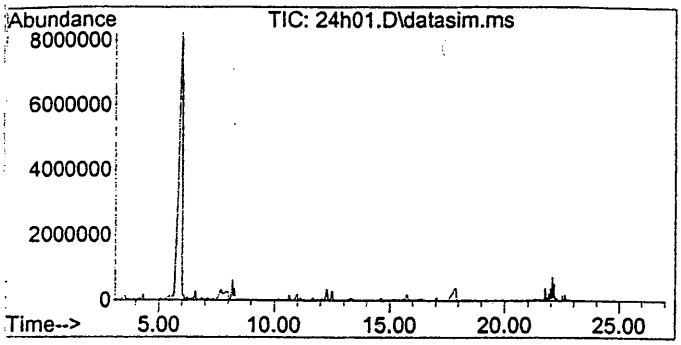
Remarques : Inhib. derivation

#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	17.03	301.3	Methyltestosterone	2,467,660	200	ng/ml
2)		12.21	438.4	Andro -D4 gluc	324,796	780	ng/ml
3)		4.14	372.3	Salbutamol -D3	1,002,310	93	ng/ml
4)		12.27	434.4	Androsterone	3,729,479	919	ng/ml
5)		12.49	434.4	Etiocholanolone	3,592,921	771	ng/ml
6)		12.65	241.2	5a3a diol	146,624	39	ng/ml
7)		12.81	241.2	5b3a diol	366,469	94	ng/ml
8)		0.00	117.1	Pregnanediol	0	0	ng/ml
9)		14.61	434.4	DHT	62,745	17	ng/ml
10)		13.71	432.4	DHEA	112,354	22	ng/ml
11)		14.27	432.4	Epitestosterone	144,227	11	ng/ml
12)		15.13	432.4	Testosterone	366,610	23	ng/ml
13)		0.00	371.3	THC -M	0	0	ng/ml
14)		0.00	369.3	Salbutamol	0	-45	ng/ml
15)		15.87	522.5	11 bOH Etiocholanolone	224,020	79	ng/ml
16)		21.32	548.5	THS	698,649	64	ng/ml
17)		22.07	636.6	THF	7,601,227	1074	ng/ml
18)		22.84	632.6	Cortisol	308,911	72	ng/ml
19)		14.63	343.3	11 Ketoetiocholanonlone	1,466,921	252	ng/ml
20)		0.00	241.2	3a-5C	0		ng/ml

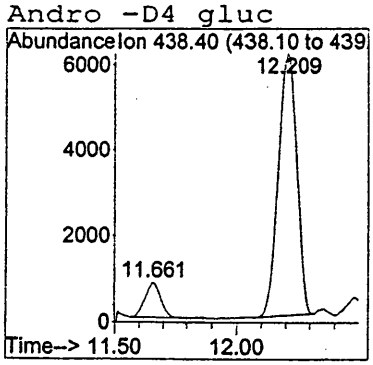
	[Surface]	[concentration]	Valeurs conventionnelles de rejet	
Ad4 glu / Si *100	13.2			< 12.9
T / ET	2.5		Rapport	> 4
Testosterone		23.4	Concentration	> 200
Epitesto		10.5	Concentration	> 200
Androsterone		919	Concentration	> 10000
Etiocholanolone		771	Concentration	> 10000
DHEA		22.0	Concentration	> 200
DHT		17.1	Concentration	> 20
Andro / Etio	1.0		Rapport	> 3
5a3a / 5b3a diol	0.4		Rapport	> 2
THC -M		0.0	Concentration	> 15
Salb/Salb D3 * 100	0	-45.0	Concentration	> 75
Cortisol / THS		1.1		
THF / THS		16.7		
11 bOH etio / THS		1.2	Pour Info (R&D)	
THF		1074		
SI/3a5c	#DIV/0!			

46

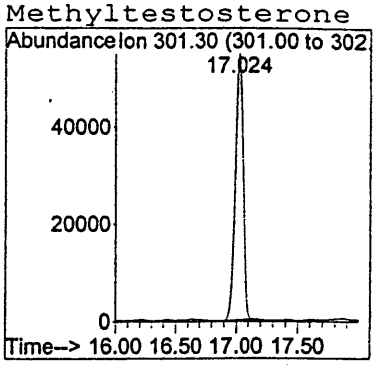
Data File: D:\MSD19\JUIL07\2407\24h01.D\da
Operator: m45p35
Date Acquired: 24 Jul 06 3:36 pm
Instrument: MSD 19
Method File: MAN06B_B.M
Sample Name: R 188/07 994080 H
Misc Info:
Vial Number : 4



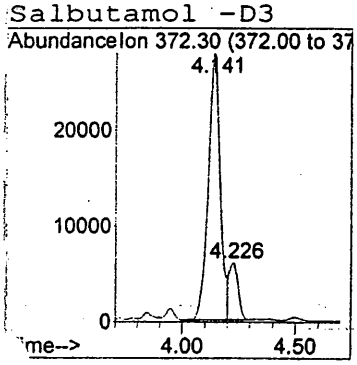
Andro -D4 gluc



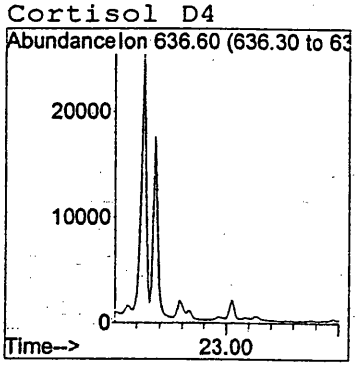
Methyltestosterone



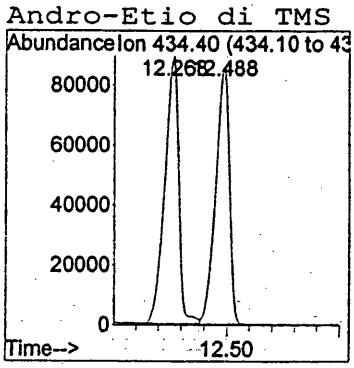
Salbutamol -D3



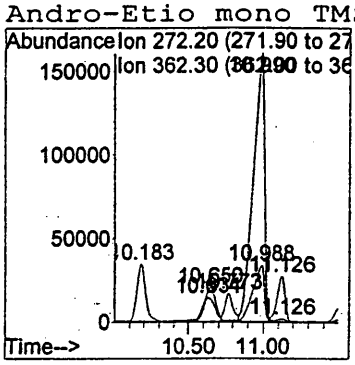
Cortisol D4



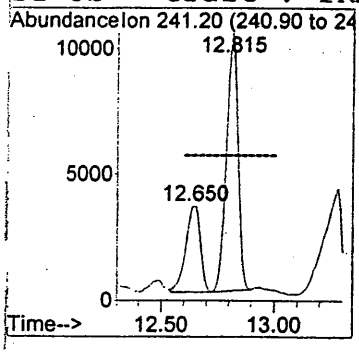
Andro-Etio di TMS

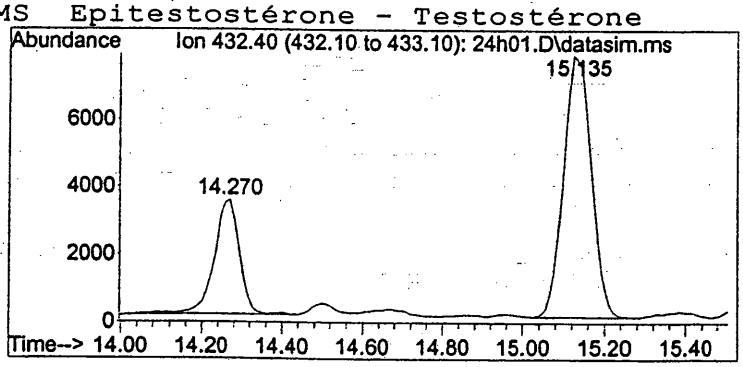


Andro-Etio mono TMS

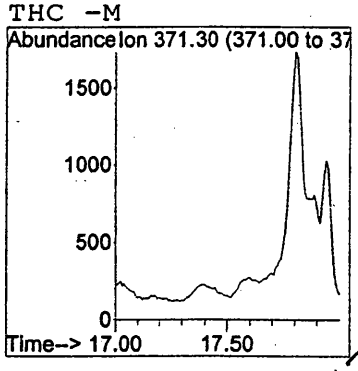


5a3a-5b3a Diol
si 5b < trait : IRMS Epitestostérone - Testostérone

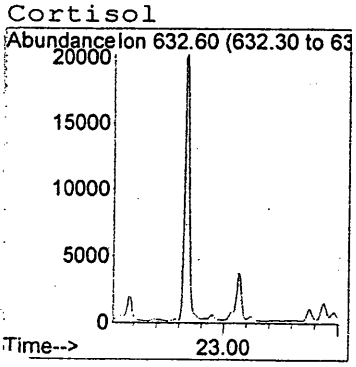




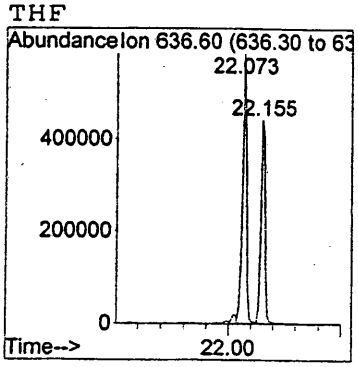
THC -M



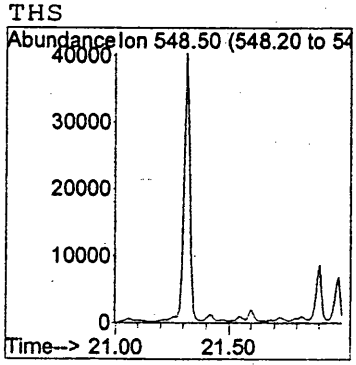
Cortisol



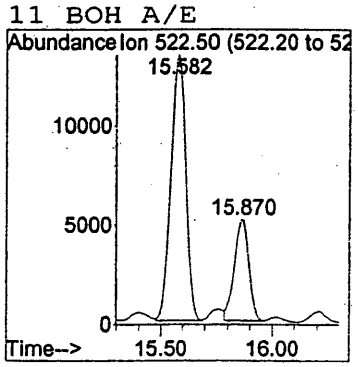
THF



THS



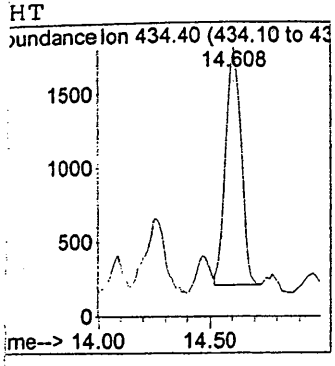
11 BOH A/E



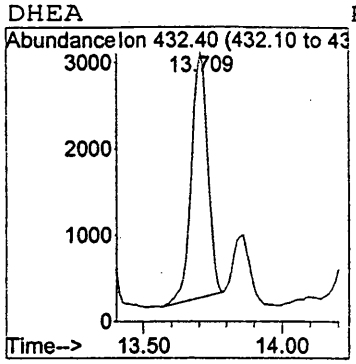
47

```
  ıe:          D:\MSD19\JUIL07\2407\24h01.D\da
 ɔerator:      m45p35
 ɔate Acquired: 24 Jul 06   3:36 pm
 Instrument:   MSD 19
 Method File:  MAN06B_B.M
 Sample Name:  R 188/07 994080 H
 Misc Info:
 Vial Number   : 4
```

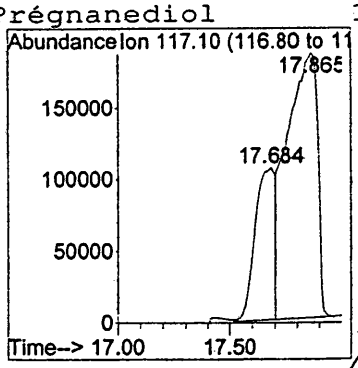
HT



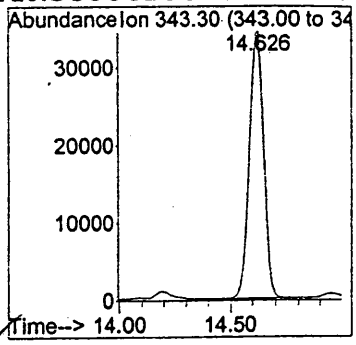
DHEA



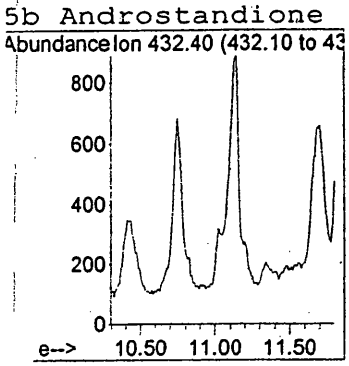
Prégnanediol



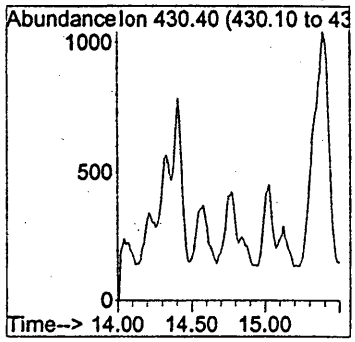
11Ketoetiocholanolone



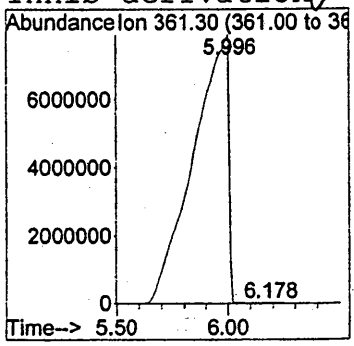
5b Androstandione



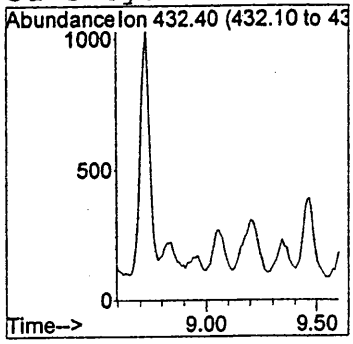
4 Androstenedione



Inhib derivation



3a-5 Cyclo



Sample 193/07 – 994171

49

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
RÉDIGÉE CONFIDENTIEL / CONFIDENTIAL

2006
17307

BUR994171

49

LNDD	ENREGISTREMENT FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS	Codification : E-Fiche_Labo-01 Version : Q Date : 18/05/2006 Page : 1 / 2

Numéro Labo: 193/07 Médecin TOUTENAERE Jean Matériel: Berlinger Nbre 3

Sport Cyclisme (UCI) En compet. (1)

Epreuve (F.I.) TDF 2006 étape n°20 à PARIS

Date(s) de Prél.: 23/07/2006 Récept° Ech. : 23/07/2006 Récept° Doc. : 23/07/2006

Date de mise en tube : 24/07/2006 Date MT EPO : 24.07.06 Date MT HES :

Observation Délai 48H EPO/HES

Ecarts EA n°: 117 Réclamat° N°:

N° :	890661	994171	995460										Operateur
Sexe:	M	M	M										V20
Filtrat (F)		F	F										Operateur
Volume	75	55	50										42
pH-bande	5	5	5										42
d-refract	1,021	1,026	1,026										42
ES06-Im	α	α	α										
ES02-Eph	-	-	-										
ES02-bbs	-	-	-										
ES04-H	(α)	α	α										
Tg/ETg	0,1	1,0	0,1										
ES05-MS2	α	α	α										
ES03-cd	α	α	α										
ES03B-LCH	α	α	α										
ES08B-PS	α	α	α										
ES08-HES													
ES07-Epo	X	x	x										
ESS01-HBO													
ESS02-TS													

Résultats :

890661 Salbutamol < seuil

Vérifié par : [illegible] Date : 26/7/06

A et B accouplés : le : par :

A et B détruits: le : par :

USADA 0466

LNDD	Enregistrement	Codification : E-Fiche_Labo-01 Version : Q Date 18/05/2006 Page : 2/2

FICHE DE SUIVI ANALYTIQUE DE RESULTAT ET DE DESTRUCTION DES ECHANTILLONS

CONCLUSIONS

Essai	Echantillon	RESULTATS ANORMAUX ET RESERVES	Date et Visa
ES.....		Présence de pH d (+/-) Dépistage : Conf	
ES.....		Présence de pH d (+/-) Dépistage : Conf	
ES.....		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH d (+/-) Dépistage : Conf :	
ES.....		Présence de à une concentration supérieure au / proche du seuil de (+/- %) pH d (+/-) Dépistage : Conf :	
ES.....		NB les résultats de(s) l'essai(s) ES ne sont pas totalement exploitables en raison d'une inhibition de due à une interférence matricielle	

Si la place est insuffisante pour conclure, utiliser E-Fiche_Labo-01Bis en complément ; dans ce cas, indiquer le nombre de feuilles utilisées

RESULTATS NORMAUX	Date et Visa	RESULTATS NORMAUX	Date et Visa
C Recherche négative pour les essais ES02C*, ES02, ES04 et ES05*	26/7/16 Ecart :	IMM RESULTAT DANS LES NORMES POUR L'ESSAI ES06	250706 Ecart :
		EPO Absence d'EPO recombinante pour l'essai ES07	260106 Ecart :
C Recherche négative pour les essais ES03 et ES03B (HA)	26/7/16 Ecart :	HES Recherche d'HEA négative pour l'essai ES08B*	26/7/16 Ecart :

dition du (des) rapport(s) d'analyse : Visa :

érification du(des) rapport(s) d'analyse par la LT et l'AQ ☑ Conv ☐ Spé Date et Visa : 28/07/06

Cet enregistrement est à archiver au département Logistique Technique

D:\MSD19\JUIL07\2407\24h04.D

Data File Path D:\MSD19\JUIL07\2407\
Data File Name 24h04.D
Operator m45p35
Acq. Method File MAN06B_B.M
Sample Name 193/07 994171 H
Misc Info
Vial Number 7
Instrument Name MSD 19
Calibration Title Calibration des stéroides endogènes
Last Calibration Update Mon Jul 24 11:26:39 2006
Fichier recal utilise 24rcl01.D

Date :
Opérateur :

C [] HC []

Résultats : Négatif []
A vérifier []

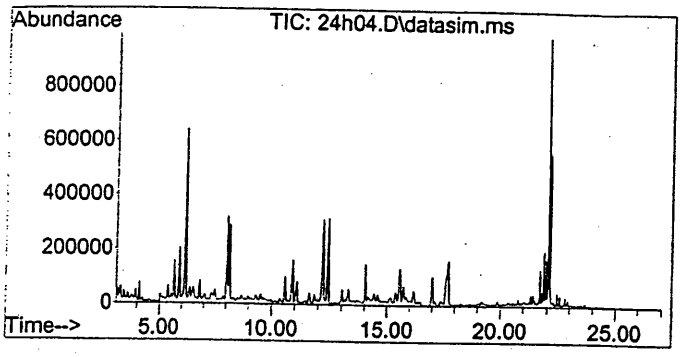
Remarques :
.....
.....

#	Peak Type	Ret Time	Signal	Name	Target Response	Amount	Units
1)	*ISTD	17.00	301.3	Methyltestosterone	2,431,784	200	ng/ml
2)		12.14	438.4	Andro -D4 gluc	388,158	946	ng/ml
3)		4.10	372.3	Salbutamol -D3	855,380	81	ng/ml
4)		12.21	434.4	Androsterone	3,205,942	802	ng/ml
5)		12.44	434.4	Etiocholanolone	3,480,691	758	ng/ml
6)		12.59	241.2	5a3a diol	62,506	17	ng/ml
7)		12.77	241.2	5b3a diol	193,979	51	ng/ml
8)		17.62	117.1	Pregnanediol	3,667,204	139	ng/ml
9)		0.00	434.4	DHT	0	0	ng/ml
10)		13.67	432.4	DHEA	84,496	17	ng/ml
11)		14.23	432.4	Epitestosterone	164,459	12	ng/ml
12)		15.10	432.4	Testosterone	171,163	11	ng/ml
13)		0.00	371.3	THC -M	0	0	ng/ml
14)		0.00	369.3	Salbutamol	0	-45	ng/ml
15)		15.85	522.5	11 bOH Etiocholanolone	272,284	97	ng/ml
16)		21.31	548.5	THS	428,455	40	ng/ml
17)		22.07	636.6	THF	11,428,833	1639	ng/ml
18)		22.83	632.6	Cortisol	422,897	99	ng/ml
19)		14.59	343.3	11 Ketoetiocholanonlone	605,369	106	ng/ml
20)		0.00	241.2	3a-5C	0		ng/ml

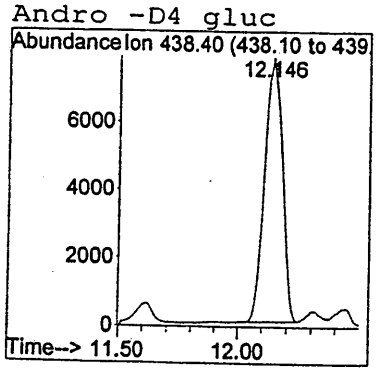
	[Surface]	[concentration]	Valeurs conventionnelles de rejet	
Ad4 glu / Si *100	16.0			< 12.9
T / ET	1.0		Rapport	> 4
Testosterone		11.1	Concentration	> 200
Epitesto		12.2	Concentration	> 200
Androsterone		802	Concentration	> 10000
Etiocholanolone		758	Concentration	> 10000
DHEA		16.8	Concentration	> 200
DHT		0.0	Concentration	> 20
Andro / Etio	0.9		Rapport	> 3
5a3a / 5b3a diol	0.3		Rapport	> 2
THC -M		0.0	Concentration	> 15
Salb/Salb D3 * 100	0	-45.0	Concentration	> 75
Cortisol / THS		2.5		
THF / THS		41.0		
11 bOH etio / THS		2.4	Pour info (R&D)	
THF		1639		
SI/3a5c	#DIV/0!			

52

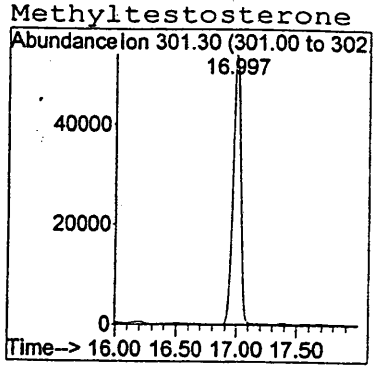
File: D:\MSD19\JUIL07\2407\24h04.D\da
Operator: m45p35
Date Acquired: 24 Jul 06 5:06 pm
Instrument: MSD 19
Method File: MAN06B_B.M
Sample Name: 193/07 994171 H
Misc Info:
Vial Number : 7



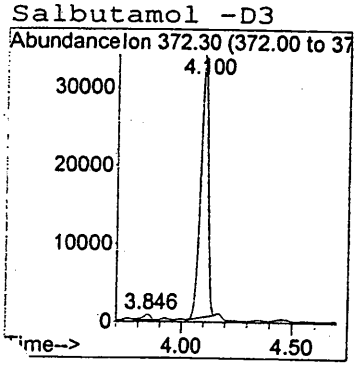
Andro -D4 gluc



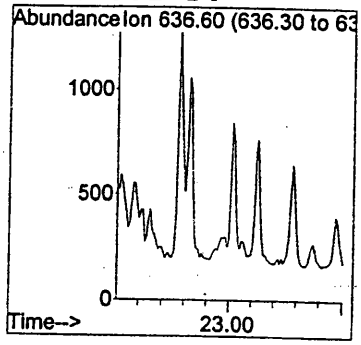
Methyltestosterone



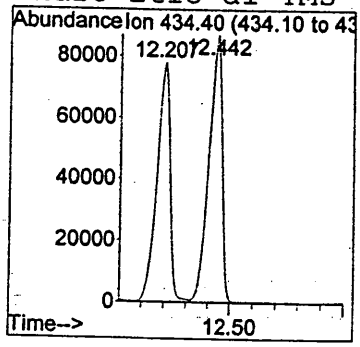
Salbutamol -D3



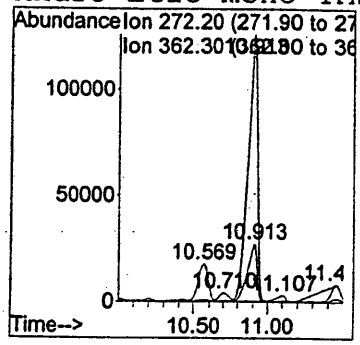
Cortisol D4



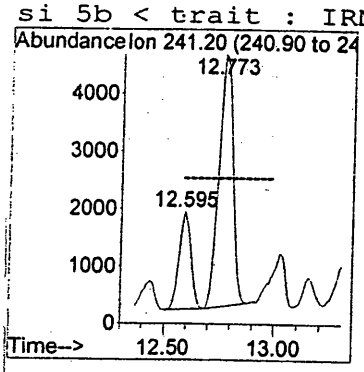
Andro-Etio di TMS

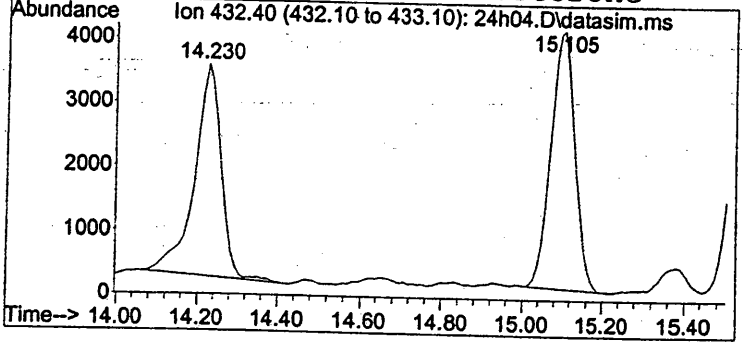


Andro-Etio mono TMS

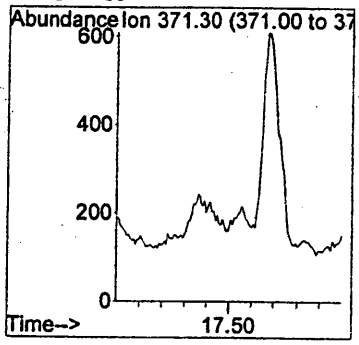


Ja3a-5b3a Diol
si 5b < trait : IRMS Epitestostérone - Testostérone

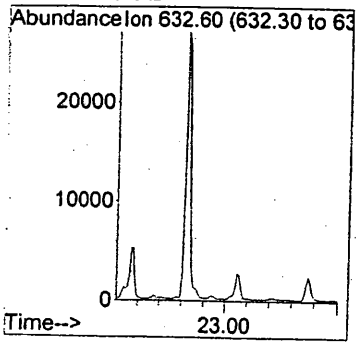




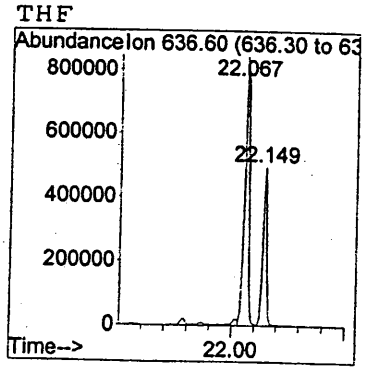
THC -M



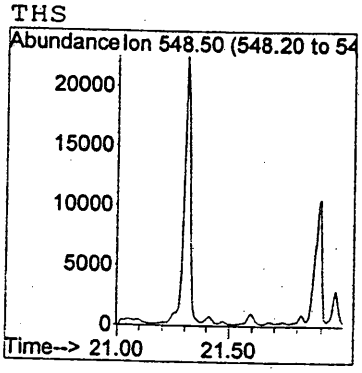
Cortisol



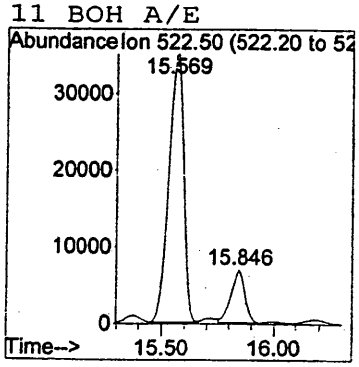
THF



THS



11 BOH A/E

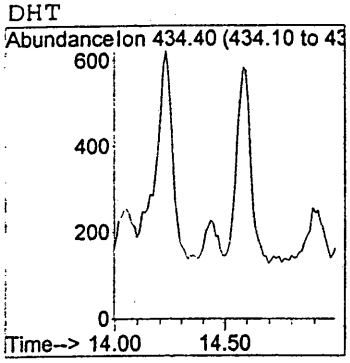


USADA 0469

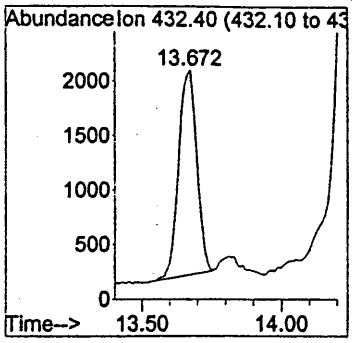
53

File: D:\MSD19\JUIL07\2407\24h04.D\da
Operator: m45p35
Date Acquired: 24 Jul 06 5:06 pm
Instrument: MSD 19
Method File: MAN06B_B.M
Sample Name: 193/07 994171 H
Misc Info:
Vial Number : 7

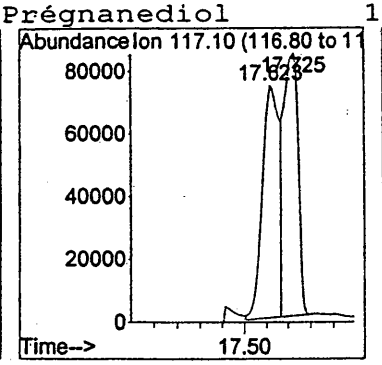
DHT



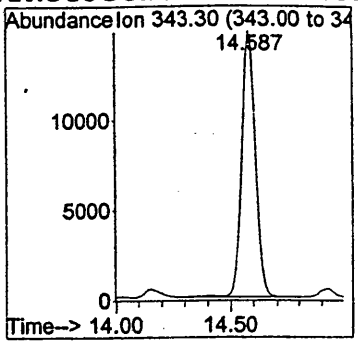
DHEA



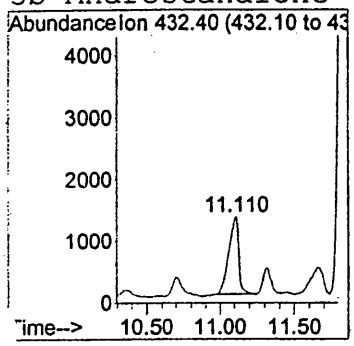
Prégnanediol



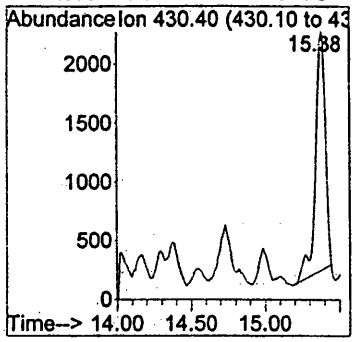
11Ketoetiocholanolone



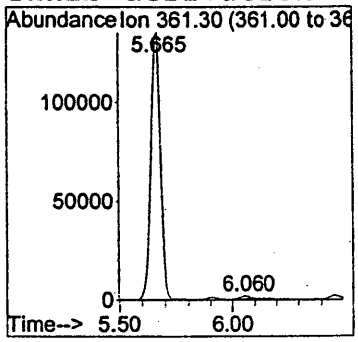
5b Androstandione



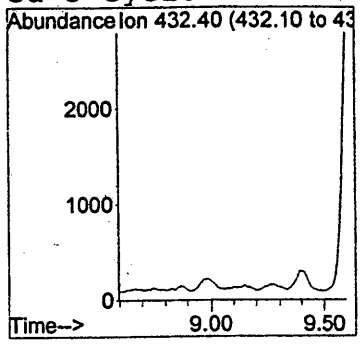
4 Androstenedione



Inhib derivation



3a-5 Cyclo



LABORATOIRE NATIONAL
DE DEPISTAGE DU DOPAGE

Please, find here the full documentation laboratory package related to sample A995474. The documentation package that related to the corresponding B sample, will be sent to USADA by UCI or by our laboratory as soon as possible, but very likely the last week of august 2006.

Kind regards,

Pr. De Ceaurriz

Director

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

Johncie Wingard

From: email direction LNDD [direction@lndd.com]
Sent: Tuesday, August 29, 2006 1:57 AM
To: Linda Barnes
Cc: 'Varin Christian - UCI'; 'Lautenschlager Delphine - UCI'; Travis Tygart; Johncie Wingard
Subject: RE : UCI Sample #995474 "B"

Dear Mrs Barnes,

Sorry for this little mistake: in the original report there is no page 105; after the page 104 there is the page 106, the number 105 was forgotten during the pagination.

With my humble apologies,

Best regards,

Adeline MOLINA
Responsable Qualité
LNDD

-----Message d'origine-----
De : Linda Barnes [mailto:lbarnes@usantidoping.org]
Envoyé : lundi 28 août 2006 22:50
À : direction@lndd.com
Cc : Varin Christian - UCI; Lautenschlager Delphine - UCI; Travis Tygart; Johncie Wingard
Objet : UCI Sample #995474 "B"
Importance : Haute

USADA received the "B" documentation package today. In our review of the package it has come to our attention that page 105 is missing.

Could you please fax or e-mail this page to us immediately.

Linda Barnes
Testing Results Manager
719-785-2024
lbarnes@usantidoping.org

Orange vous informe que cet e-mail a été contrôlé par l'anti-virus mail.
Aucun virus connu à ce jour par nos services n'a été détecté.

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

Johncie Wingard

From: Travis Tygart
Sent: Thursday, October 19, 2006 8:20 AM
To: Philippe Verbiest; Gripper Anne - UCI
Cc: Johncie Wingard
Subject: Landis Case.
Attachments: USADA_20061019_071100.pdf

Dear Philippe and Anne-

Based on our experience in the Hamilton case, we think it would be best for USADA, UCI and WADA if we had a single hearing before CAS in the Landis case instead of an AAA hearing and then a new CAS hearing. What we learned in Hamilton is that Mr. Jacobs will raise all of the same arguments in the second hearing and will come up with new arguments to replace those that were entirely discredited. He will also come up with a whole new suite of expert witnesses to replace those that we are able to discredit at the first hearing. He has already made a lengthy document request to UCI in the Landis case so it is clear that you all will not be able to avoid involvement.

Knowing that both UCI and WADA would have to consent, we ran the idea of a single hearing before Mr. Jacobs (noting that that was the route his clients chose in Montgomery and Gaines) and in response we have received the attached letter.

It would seem to me that in terms of overall time, expense and pursuit of the truth, a single hearing would be the best way to go. Let's discuss this after you have had a chance to consider. We have also alerted WADA to this matter.

Please call if you have any questions or concerns but can we speak in the next day or so to finalize?

Sincerely,

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

LAW OFFICES OF HOWARD L. JACOBS

October 16, 2006

VIA FACSIMILE 719-785-2001 AND REGULAR MAIL

Travis Tygart
USADA
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: USADA v. Floyd Landis
AAA Case No. 30 190 00847 06

Dear Travis:

As a follow-up to your proposal to proceed directly to CAS in the above-referenced matter, Floyd Landis is agreeable to that proposal under the following conditions:

1. The USADA Protocol and AAA Supplemental Procedures for the Arbitration of Olympic Sport Doping Disputes shall supersede any other applicable rule that is inconsistent with said rules;
2. The CAS Rules Applicable to the Ordinary Arbitration Procedure shall govern, except as modified by point #1 above;
3. The Arbitration Hearing shall be an open public hearing held at the Pepperdine University School of Law in Malibu, California.
4. The CAS decision shall be final and binding on all parties and shall not be subject to further review or appeal; and
5. The above terms will be incorporated into a written agreement that will be agreed to in writing by Floyd Landis, USADA, UCI, WADA and CAS.

I look forward to your response.

Very truly yours,

Howard L. Jacobs

cc: Floyd Landis (via e-mail)

5210 Lewis Road
Suite 5
Agoura Hills, CA 91301
USA

PHONE (818) 292-8735
FAX (818) 292-8736
ALT. FAX (818) 942-6079
E-MAIL howard.jacobs@yahoo.com
WEB SITE http://www.athleteslawyer.com

TAB 42

June 21, 2001

To: Clients of the UCLA Olympic Laboratory

Regarding: **Carbon Isotope Ratio measurements, Update 3**

This letter is to update you on our carbon isotope ratio method and to explain the wording we use in our current reports.

DIOL ASSAY

Requests: Currently when you request a CIR analysis we perform the "Diol" assay. The procedure is to request a CIR analysis by fax or email and to provide the sample number. We will check the original data on the sample and determine if the analysis is likely to be successful. If not, we will advise you that we do not believe the analysis will be successful and we may suggest an alternative approach. In order to enhance our understanding of the analysis we also ask that you give us all sample numbers of prior samples from the same athlete that were analyzed at UCLA.

The "Diol' assay determines the carbon isotope ratio (delta value) of two diol metabolites of testosterone which we refer to as M1 and M2, and one metabolite of a testosterone precursor (Pdiol). [See the metabolic map attached.] The assay determines the ratio of $^{13}C/^{12}C$ for each of these three steroids. The units are usually called "delta units". In addition to the delta values for these steroids, two other types of measurements are calculated. The first is the ratio of the metabolites to the precursor. Two ratios are calculated: M1/Pdiol and M2/Pdiol. The second is the difference between the metabolites and the Pdiol: M1-Pdiol and M2-Pdiol.

Endogenous reference compound: The Pdiol serves as an endogenous reference compound (ERC). Since it is a metabolite of a precursor (see map) in the testosterone metabolic scheme, its delta value does not change when testosterone is administered. In the typical positive case, the delta values of M1 and M2 are low and the delta value of Pdiol is within the normal range. In negative cases, all three diols have similar delta values. The reporting terminology will be: Positive, Negative, or Indeterminate.

A POSITIVE report means that the delta values for both M1 and M2 are at least three standard deviation (SD) units less than the mean (average) of a group of 73 normal males, and the delta value for Pdiol is within 3 SD of the mean of normal males. In addition the two ratios (M1/Pdiol and M2/Pdiol) and the two differences (M1-Pdiol and M2-Pdiol) are more than 3 SD from the range of normal values. These criteria are very conservative because all must be met for the sample to be declared positive.

A NEGATIVE report means that all three delta values, the two difference scores, and the two ratios are within the normal range.

An INDETERMINATE report means that we were not able to obtain definitive data. The most common reasons for this are insufficient sample volume and low concentrations of the steroids. Since there are several criteria for a positive report, it is also possible that a sample will have one ratio and one difference score that are normal and the other ratio and difference score will be abnormal. Other combinations of results are possible. Based on our current understanding of the theory underlying CIR measurements, we believe that in these mixed cases that we classify as indeterminate, some of the molecules of M1 or M2 are derived from pharmaceutical testosterone (or another exogenous steroid which is metabolized to

testosterone) and some of the molecules are from natural (endogenous) testosterone. This is expected when the body contains a mixture of pharmaceutical and natural testosterone and metabolites. This situation is likely to occur at the later times in the curve that relates delta values to time since drug administration. As our clients gain further understanding of the CIR analysis, you may have other opinions about how we report mixed cases.

SAMPLES FROM OTHER LABORATORIES

Occasionally we are asked to perform a CIR analysis on a sample that was originally processed at another sample. We are not comfortable with this for four reasons: 1) we do not know the details of handling and storage of the sample in the other laboratory, 2) we do not have any control over the chain-of-custody until the sample arrives at UCLA, 3) we do not have screening data (T/E, testosterone concentration, etc.) obtained at UCLA therefore we cannot adequately plan the analysis, and 4) we do not have control samples that were handled in the same way. We do not have any reason to believe that the CIR analysis is affected by storage conditions, time factors, or temperature nevertheless we cannot be absolutely certain that our in-house control data applies to such samples. In the future we will decline to perform the CIR analysis unless there is sufficient sample volume to perform both a steroid analysis and a CIR analysis.

ANDRO & ETIO ASSAY

Under certain circumstances we also offer the Andro & Etio assay. The advantages are that it is simpler to perform, less expensive, and it almost always yields definitive results. The disadvantage is that there is no Pdiol to serve as an ERC, therefore we are not able to calculate ratios or difference scores. Most clients seem to prefer the DIOL assay, however we suspect that in the future the Andro&Etio assay will be developed to the point where the lack of an ERC will not be a disadvantage. At this time we recommend the "diol' assay. Occasionally we will advise you that it is not possible to perform the DIOL assay and that the only CIR option is the Andro & Etio assay.

GENDER

At the present time we do not recommend performing the CIR analysis on samples from females. If you still wish to have the analysis we will determine if it is feasible to proceed based on the existing data from the athlete.

EPITESTOSTERONE

We have been working on a CIR assay for epitestosterone and we have recently presented our findings at a national meeting. There is no peer reviewed publication at this time and it will take about a year to prepare a manuscript and get it published. Nevertheless, at your request we will conduct the epitestosterone analysis on urine samples reported "epitestosterone > 200 ng/ml".

TURN-AROUND-TIME

In an attempt to keep the costs down we are performing the analyses one week per month. Typically this is the fourth week of the month. It takes about a week to complete the analysis, calculate the results, and the issue the report.

COSTS

In 2000 we performed about 20 CIR analyses and charged $600./sample. Our costs were approximately $40,000. We made up the loss with CIR research funds. I was reluctant to use research funds for this service function, however I felt that it was also important to release the test. In addition, there is substantial research value in the results that we obtain on your samples because we are able to correlate the CIR data with the T/E and other data from basic GC-MS steroid analysis. This year we are performing more analyses and still charging $600. However, it is evident that we will not have $40,000. of income from the analysis, even though the request rate is substantially higher this year. We have exhausted the research funds, therefore in the Fall we may have no choice but to substantially raise the price.

FEEDBACK

We recognize that the CIR analysis is evolving and that there are many different ways to manage, treat, and report the data. We would be delighted to have your comments on these issues.

Sincerely,

Don H. Catlin, M.D.

cc:
NFL (John Lombardo, M.D., Bryan Finkle, Ph.D. Adolpho Birch, Harold Henderson, Peter. Ruocco, Dennis Curran)
USADA (Terry Madden, Larry Bowers, Ph.D.)
USOC (Richard Young, Gary Johansen)
NCAA (Frank Uryasz, Andrea Wickerham)
International group

encl: Metabolic map June 22, 2001

Explanation of the Carbon Isotope Ratio Method in Doping Control

Don H. Catlin, M.D.
UCLA Olympic Analytical Laboratory

Atoms and molecules: Everything in nature is made of molecules and most molecules are made of atoms of carbon, hydrogen, and oxygen. Atoms are nature's building blocks and carbon is a dominant atom in the human body.

Food-chain: The human food-chain begins with plants. Plants get their carbon by photosynthesis - they take in or "fix" the carbon dioxide in the air. Animals eat plants. Many humans eat plants and animals. Vegetarians eat only plants. Ultimately all carbon atoms in the human body are derived from the carbon dioxide in the air.

The instrument: Most of the carbon all around is ^{12}C (carbon 12), but a small amount is ^{13}C, a different isotope, heavier by one extra neutron. Roughly 1.1% of all carbon is ^{13}C, but different compounds contain more or less ^{13}C. These small differences in ^{13}C content can be measured with an instrument known as GC/C/IRMS. Our GC/C/IRMS method is designed to determine differences in the ratio of ^{13}C to ^{12}C in biological molecules such as testosterone, its precursors, and its metabolites. The units of measurement are called delta units (δ).

Nature's label: Through a fortuitous quirk of nature, there is a measurable difference in ^{13}C content between biosynthetic and pharmaceutical testosterone. This is because they arise from different pathways. Biosynthetic testosterone is made in the human body from cholesterol. Pharmaceutical testosterone is synthesized from plant products (soy and others). It contains less ^{13}C than natural or endogenous testosterone, therefore its $^{13}C/^{12}C$ ratio is lower. By measuring just how much lower, if at all, the GC/C/IRMS method determines whether the testosterone is from pharmaceutical or endogenous sources. Urinary steroids with a low delta value originate from pharmaceutical steroids.

Role of steroid metabolism: Endogenous testosterone is biosynthesized from cholesterol via a complicated pathway which can be abbreviated as cholesterol → pregnenolone → progesterone → androstenedione → testosterone. After testosterone is biosynthesized, it is metabolized or broken down to DHT, androsterone, etiocholanolone, 5α-diol and 5β-diol. All these steroids appear in human urine. If an individual takes pharmaceutical testosterone, the testosterone in the urine will be labeled with a low $^{13}C/^{12}C$ ratio and so will all the metabolites of testosterone. Thus by determining the $^{13}C/^{12}C$ ratio of urine 5α-diol and 5β-diol (testosterone metabolites), the test is determining whether the metabolites came from natural or pharmaceutical testosterone. 5β-pdiol is not involved in testosterone metabolism, therefore it is not affected by taking testosterone, and that makes it a good endogenous reference compound (ERC).

Different types of GC/C/IRMS analysis: The CIR test can be used in several different ways to detect the use of many different steroids. The selection of which steroids to measure the $^{13}C/^{12}C$ ratio on is complex and depends on urine volume, costs, time, and other factors. In the form of the CIR test used for this report, the measurement is done on 5α-diol, 5β-diol, and ERC. If the value for 5α-diol is between about -24.5 and -27.9 delta, and if it is similar to the ERC value, it is normal. If it is low (more negative than - 27.9 and more negative than the ERC value), this indicates that the individual submitting the urine used testosterone, androstenedione, DHT, or DHEA. Most doping control programs prohibit the use of all these steroids.

Travis Tygart

From: Don Catlin [dcatlin@ucla.edu]
nt: Friday, May 02, 2003 1:08 PM
o: Travis Tygart; Larry Bowers
Cc: Ryan Connolly (E-mail); Brian Ahrens
Subject: request for CIR analysis



cir client interpretation.rtf ...

Dear Larry and Travis:

Enclosed is our protocol for performing cir analyses. We follow this protocol There are several issues that need to be sorted out before a decision can be made.

We do not offer the assay on a routine basis. If you wish to have it next Monday, as opposed to the once a month schedule noted in section 'turn-around-time', we we will attempt to accomodate your stat request but it will depend on the availabilty of key staff. I will write again once I know the answer.

The fee will be $600. and we need an email from you noting that USADA will be responsible for the fee. We cannot be responsible for collecting from IAAF.

You need to tell us if the analysis should be performed on the A or B.

You also need to tell us whether you wish to have the Diol or the A&E analysis. The concentraion of the 5a and 5b diol are low thus we need 30 mL for the Diol assay. We recommend the diol assay.

need 15 mL for the TE and another 15 mL in reserve in case we need to repeat it. There are only 50 mL total thus we do not have enough. We need to make decisions on volumes. If you ask for the cir on the A leftovers, that will solve the problem.

We need the prior TEs as described in item 'Requests'.

It will take at least two days for the cir analysis to be completed. The athlete will not be able to watch both assays simultaneously due to space issues.

Just so we cover all the potential points of conflict, if the athlete chooses to observe the entire procedure, they could be here for 48 hours. We will provide an escort 8h per day for two days. If more time is required we will have to bill USADA for the time.

All things considered, performing a TE and a CIR simultaneously on one sample on short notice will not be simple, thus it would be better to schedule them sequentially.

Don

UNIVERSITY OF CALIFORNIA, LOS ANGELES UCLA

BERKELEY · DAVIS · IRVINE · LOS ANGELES · RIVERSIDE · SAN DIEGO · SAN FRANCISCO SANTA BARBARA · SANTA CRUZ

UCLA OLYMPIC ANALYTICAL LABORATORY
DEPARTMENT OF PHARMACOLOGY
UCLA SCHOOL OF MEDICINE
2122 GRANVILLE AVENUE
LOS ANGELES, CALIFORNIA 90025
PHONE (310) 825-2635
FAX (310) 206-9077

May 19, 2003

To: Clients of the UCLA Olympic Laboratory

Regarding: **Carbon Isotope Ratio measurements, Update 4**

This letter is to update you on the availability of carbon isotope ratio analyses. We have resumed research on the carbon isotope ratio analysis, therefore we are now able to perform CIR analyses on service samples.

REQUESTS

Currently when you request a CIR analysis we perform the "DIOL" assay. The procedure is to request a CIR analysis by fax or email and to provide the sample number. We also request that you provide the sample numbers corresponding to prior samples that we have analyzed from the same athlete.

We will check the original data on the sample (primarily concentration of diols) and determine if the analysis is likely to be successful. If the DIOL assay is not likely to succeed we will perform the ANDRO & ETIO assay. If you request that we attempt the DIOL assay anyway we will do so and report the results. If the DIOL assay yields an indeterminate result, you will have the option of requesting the ANDRO & ETIO assay.

DIOL ASSAY

The DIOL assay determines the carbon isotope ratio (delta value) of two diol metabolites of testosterone which we refer to as M1 and M2, and one metabolite of a testosterone precursor (Pdiol). Please review prior updates, particularly update 3 dated June 21, 2001, for the details of the analysis and the interpretation. The reporting categories remain: positive, negative, and indeterminate.

ANDRO & ETIO ASSAY

The advantages are that compared to the DIOL assay, the ANDRO & ETIO assay is simpler to perform, less expensive, and it almost always yields definitive results. The disadvantage is that there is no Pdiol to serve as an endogenous reference compound (ERC), therefore we are not able to calculate ratios or difference scores. Most clients prefer the DIOL assay, however we suspect that in the future the Andro & Etio assay will be developed to the point where the lack of an ERC will not be considered a disadvantage.

SAMPLES FROM OTHER LABORATORIES - no change in policy, see prior updates.

GENDER

At the present time UCLA does not have a complete database on CIR values for females. The concentrations of diols are generally lower in samples obtained from females and we have not yet developed a method for low-level diol samples. If you request a CIR analysis on a female sample we will attempt the DIOL analysis as described above. If the assay is not successful you may then request the ANDRO & ETIO assay. Or, you may request the ANDRO & ETIO analysis directly.

EPITESTOSTERONE

The assay development has been completed and we have published a paper in a peer-reviewed journal. [Aguilera R, Hatton CK, Catlin DH. Detection of doping with epitestosterone by isotope ratio mass spectrometry. Clin Chem 2002;48:629-636] The assay is recommended for samples reported as "epitestosterone > 200 ng/ml".

TURN-AROUND TIME

In an attempt to keep the costs down we are performing the analyses one week per month. Typically this is the fourth week of the month. It takes about a week to complete the analysis, calculate the results, and the issue the report.

COSTS

In 2000 we performed about 20 CIR analyses and charged $600/sample. Our costs were approximately $40,000. We had to discontinue the CIR analyses in 2001 due to continuing losses and lack of research support. Based on our best estimate of the number of CIR analyses we expect to perform in the next year we have established the following fee schedule assuming that the work is performed only one week per month:

DIOL assay	400.
ANDRO & ETIO assay	350.
Epitestosterone assay	350.

In the event that you cannot wait for the monthly analysis we can do the analyses on a stat basis for the above fee plus 950. per batch.

FEEDBACK

We recognize that the CIR analysis is evolving and that there are many different ways to manage, treat, and report the data. We would be very pleased to receive your comments on the matters described herein.

Sincerely,

Don H. Catlin, M.D.
Director

Michael H. Sekera
Scientific Director

United States Anti-Doping Agency
1330 Lake Plaza Drive, Suite 260
Colorado Springs, CO 80906
Phone: (719) 785-2000
Fax: (719) 785-2001
usada@usantidoping.org

Fax

To: Dr. Catlin or Michael Sekera or Ana Reyes **From:** Linda Barnes

Fax: 310-206-9077 **Date:** July 26, 2006

Phone: 719-785-2024 **Pages:** 2, including cover sheet

Re: Request for T/E Ratio Information **CC:**

☐ **Urgent** ☐ **For Review** ☐ **Please Comment** ☐ **Please Reply** ☐ **Please Recycle**

URGENT

We have an athlete with that has been reported as having an elevated T/E ratio and a positive CIR analysis from another laboratory. We will need to do a follow up investigation on this athlete.

As part of our follow-up investigation of this athlete, we will need to obtain additional information on the athlete's prior samples. I have attached a list of the sample #'s and collection dates. **We need T/E ratio as well specific gravity and pH on each sample, if possible.**

Also, in continuing to evaluate the best method to assess elevated T/E ratio tests, we would like to be able to evaluate the steroid profile, as reported by Donike. While we understand the measurement of androsterone, etiocholanolone, 11β-hydroxyandrosterone, 11β-hyroxyetiocholanolone in the screening and confirmation methods and epitestosterone and testosterone in the screening method are not quantitative, some review panels and international federations have found them of benefit.

Thank you for the extra effort I know this entails.

Best regards,

Linda Barnes, Testing Results Manager

CONFIDENTIALITY NOTE: The information contained in this facsimile transmittal sheet and document(s) that follow are for the exclusive use of the addressee and may contain confidential, privileged and nondisclosable information. If the recipient of this facsimile is not the addressee, or a person responsible for delivering this facsimile to the addressee, such recipient is strictly prohibited from reading, photocopying, distributing or otherwise using this facsimile transmission, or its contents, in any way. If the recipient has received this facsimile transmission in error, please call us immediately and return the facsimile transmission to us via the United States Postal Service. Thank you.

Date	Test Type	Sample Number	Outcome	Result
4/10/2006	Out of Competition	1504999	Completed	Negative
1/6/2006	Out of Competition	493091	Completed	Negative
11/5/2005	Out of Competition	493084	Completed	Negative
10/7/2005	Out of Competition	491607	Completed	Negative
1/8/2005	Out of Competition	485155	Completed	Negative
11/26/2003	Out of Competition	476315	Completed	Negative
12/8/2002	Out of Competition	466193	Completed	Negative

Linda Barnes

From: Linda Barnes
Sent: Tuesday, August 01, 2006 1:15 PM
To: 'Ana Reyes'
Cc: Travis Tygart
Subject: Request for T/E Ratio Info

Ana

I just re-faxed a request for steroid concentration info that I sent to the lab last week. Can you please check on the status of this request for me?

Thanks for your help.

Linda Barnes

Testing Results Manager

719-785-2024

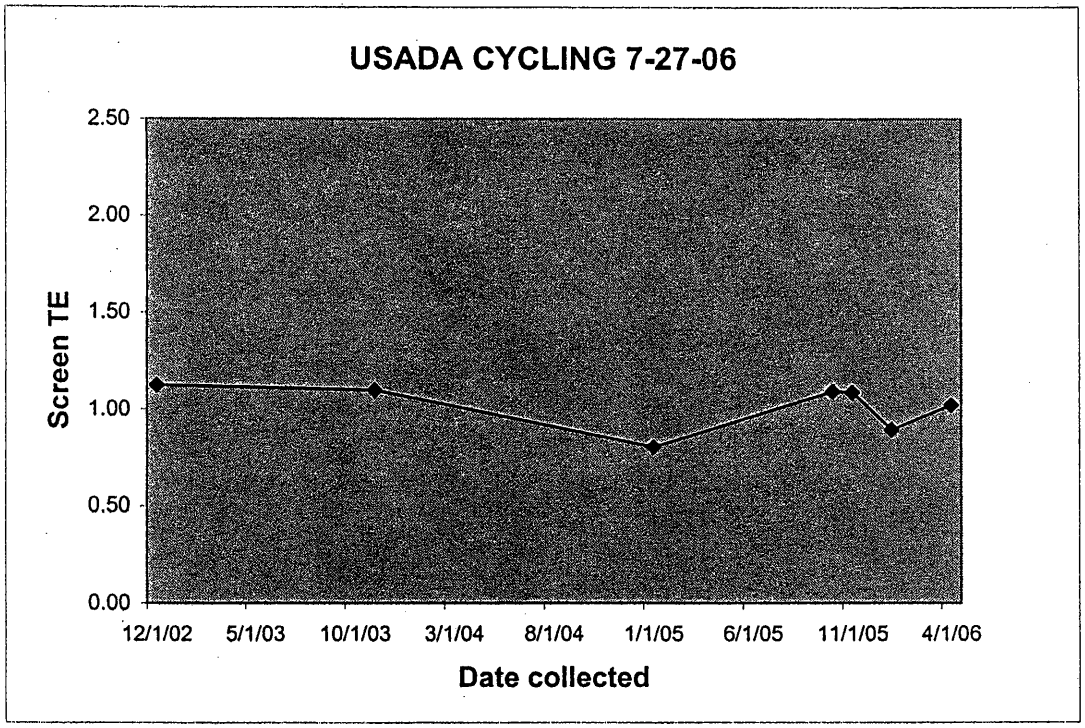
lbarnes@usantidoping.org

Johncie Wingard

m: Don Catlin [dcatlin@ucla.edu]
Sent: Tuesday, August 01, 2006 5:17 PM
To: Travis Tygart
Subject: Data you requested
Attachments: USADA TE ratio Cycling 7-27-06.xls

USADA ELEVATED TE RATIO REQUEST - July 27, 2006

BOTNUM	LABCODE	SPORT	GENDER	PHL	SGL	COLLECT	te	TESTX	EPITX	ANDX	ETIX	DIOL5AX
466193	YWI08	CYCLING	M	7.00	1.020	12/08/02	1.13	16.1	15	1,692	1,676	45
476315	1ER03	CYCLING	M	5.00	1.023	11/26/03	1.10	14	14.7	859	1,633	37
485155	4JF04	CYCLING	M	7.50	1.012	01/08/05	0.80	17.2	23.8	933	1,310	39
491607	63L03	CYCLING	M	5.00	1.026	10/07/05	1.09	40.6	41.9	1,898	2,459	138
493084	6W903	CYCLING	M	5.00	1.018	11/05/05	1.08	29.8	30	1,367	1,668	75
493091	7YG09	CYCLING	M	5.50	1.025	01/06/06	0.89	29.5	33.7	1,284	3,985	40
1504999	8YZ08	CYCLING	M	7.50	1.024	04/10/06	1.02	53.6	53	1,525	3,745	48



DIOL5BX

169
153
98
435
255
253
398

USADA 0488

Johncie Wingard

From: Philippe Verbiest [philippe.verbiest@skynet.be]
Sent: Tuesday, August 08, 2006 12:00 PM
To: Travis Tygart
Subject: Landis

Dear Travis,

A fax was sent to you by C. Varin concerning Floyd Landis. This fax contains an error.

Please disregard and destroy this fax. A new fax will be sent tomorrow.

Regards,

Ph. Verbiest

Johncie Wingard

From: Philippe Verbiest [philippe.verbiest@skynet.be]
Sent: Wednesday, August 09, 2006 9:21 AM
To: Linda Barnes
Cc: Travis Tygart
Subject: RE: Fax received from UCI

Dear Ms Barnes

Thank you and best regards,

Ph. Verbiest

From: Linda Barnes [mailto:lbarnes@usantidoping.org]
Sent: mardi, 8. août 2006 22:37
To: philippe.verbiest@skynet.be
Subject: Fax received from UCI

Mr. Verbiest

Travis Tygart asked that I send you an e-mail confirming that I have shredded the documents faxed earlier to USADA today. No copies were kept and nothing was sent to any other entity.

Best regards,

Linda Barnes
Testing Results Manager
719-785-2024
lbarnes@usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

INTERNATIONAL CYCLING UNION

USADA
Mr. Travis Tygart
Senior Managing Director and
General Counsel
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906-4651
USA

First by fax: + 1 719 785 20 01

Aigle, 9 August 2006
Ref: Anti-doping / CV / pq

Dear Mr. Tygart,

Please find enclosed:

1) a table with the T/E ratio's of Floyd Landis as from September 1999 ("0" means that no T/E measurement was made); and
2) a copy of the corresponding laboratory reports of 2005 and 2006.

Sincerely Yours,

Anti-Doping Services:

Christian VARIN, Manager

Encl.

CH 1860 Aigle / Switzerland
☎+41 24 468 58 11 fax +41 24 468 58 12
www.uci.ch

Nom et Prénom	Date (AD)	Course	Laboratoire	Pays course	No éch	T/E
LANDIS FLOYD	08.09.1999	TOUR DE L'AVENIR	PARIS	FRANCE	186671	2
LANDIS FLOYD	18.09.1999	GP DES NATIONS	PARIS	FRANCE	185105	2.1
LANDIS Floyd	26.01.2000	TOUR DE LANGKAWI ETAPE 1	PENANG	MALAISIE	055383	0
LANDIS Floyd	30.04.2000	VUELTA CICLISTA A LA RIOJA	MADRID	ESPAGNE	311370	2.1
LANDIS Floyd	24.08.2000	TOUR POITOU CHARENTES	PARIS	FRANCE	217241	1.5
LANDIS Floyd	26.08.2000	TOUR POITOU CHARENTES	PARIS	FRANCE	217243	0.9
LANDIS Floyd	01.04.2001	CRITERIUM INTERNATIONAL	PARIS	FRANCE	216923	1.1
LANDIS Floyd	26.06.2001	ROUTE DU SUD	PARIS	FRANCE	210038	1.1
LANDIS Floyd	16.06.2002	CRITERIUM DU DAUPHINE LIBERE	PARIS	FRANCE	176593	1.2
LANDIS Floyd	26.07.2003	TOUR DE FRANCE	PARIS	FRANCE	808345	0.9
LANDIS Floyd	15.08.2003	VUELTA CICLISTA A BURGOS	MADRID	ESPAGNE	336873	1.5
LANDIS Floyd	22.02.2004	VOLTA AO ALGARVE	LISBONNE	PORTUGAL	277821	0
LANDIS Floyd	09.03.2004	PARIS - NICE	PARIS	FRANCE	289130	0.9
LANDIS Floyd	04.09.2004	VUELTA A ESPAÑA	MADRID	ESPAGNE	342599	1.7
LANDIS Floyd	11.09.2004	VUELTA A ESPAÑA	MADRID	ESPAGNE	342856	2
LANDIS Floyd	12.09.2004	VUELTA A ESPAÑA	MADRID	ESPAGNE	342850	1.4
LANDIS Floyd	13.09.2004	VUELTA A ESPAÑA	MADRID	ESPAGNE	348369	1.3
LANDIS Floyd	14.09.2004	VUELTA A ESPAÑA	MADRID	ESPAGNE	347883	1

LANDIS Floyd	21.04.2005	TOUR DE GEORGIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	920460	0
LANDIS Floyd	22.04.2005	TOUR DE GEORGIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	920462	0
LANDIS Floyd	17.07.2005	TOUR DE FRANCE	PARIS	FRANCE	874535	2.2
LANDIS Floyd	22.02.2006	TOUR OF CALIFORNIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	951826	0
LANDIS Floyd	23.02.2006	TOUR OF CALIFORNIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	951875	0
LANDIS Floyd	24.02.2006	TOUR OF CALIFORNIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	951828	0
LANDIS Floyd	25.02.2006	TOUR OF CALIFORNIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	951866	0
LANDIS Floyd	26.02.2006	TOUR OF CALIFORNIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	951872	0
LANDIS Floyd	08.03.2006	PARIS - NICE	PARIS	FRANCE	876013	1.5
LANDIS Floyd	09.03.2006	PARIS - NICE	PARIS	FRANCE	874285	1.5
LANDIS Floyd	10.03.2006	PARIS - NICE	PARIS	FRANCE	872364	1.3
LANDIS Floyd	11.03.2006	PARIS - NICE	PARIS	FRANCE	872357	1.6
LANDIS Floyd	12.03.2006	PARIS - NICE	PARIS	FRANCE	876004	1.6
LANDIS Floyd	20.04.2006	TOUR DE GEORGIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	951792	0
LANDIS Floyd	21.04.2006	TOUR DE GEORGIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	951789	0
LANDIS Floyd	22.04.2006	TOUR DE GEORGIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	951787	0

LANDIS Floyd	23.04.2006	TOUR DE GEORGIA	LOS ANGELES	ETATS-UNIS D'AMERIQUE	951790	0
LANDIS Floyd	08.07.2006	TOUR DE France	PARIS	FRANCE	994362	2.8
LANDIS Floyd	11.07.2006	TOUR DE France	PARIS	FRANCE	994203	1.3
LANDIS Floyd	13.07.2006	TOUR DE France	PARIS	FRANCE	994277	2.5
LANDIS Floyd	14.07.2006	TOUR DE France	PARIS	FRANCE	994216	2
LANDIS Floyd	18.07.2006	TOUR DE France	PARIS	FRANCE	994075	1.8
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
LANDIS Floyd	22.07.2006	TOUR DE France	PARIS	FRANCE	994080	2.5
LANDIS Floyd	23.07.2006	TOUR DE France	PARIS	FRANCE	994171	1

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS, FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP ROAD
CA 92562 MURRIETA
ETATS UNIE (USA)

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 00202721 USA 1975 1014

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : USA
Date de naissance / Birth Date : 1975/10/14
Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : UCI - FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 2D ETE ET

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 23-07-2006
Heure de remise de la notification : 17.30 H.
Lieu du contrôle / Place of test : PARIS, WHITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested : LANDIS, FLOYD

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VAN GESTEL, JAN

Nom et prénom du médecin / Name of doctor : (en toutes lettres) TOLLENAERE, BRUNO

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 18.45 h
Date du prélèvement / Sampling date : 23-07-2006
Heure du prélèvement / Sampling time : 18.50 h

Identification / Identification :
Licence / Licence : ☒
Carte d'identité / ID : ☐
Passeport / Passport : ☐
Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994171

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 110 mL

Densité / Specific Gravity : 1,020
pH / pH : 6.0

Échantillon d'urine insuffisant / Insufficient urine sample

cellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐ (AUT)

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

15/05/2006 DEXAMETHASONE Ecrase Duree 6.3jours
15/05/2006 Methyl prednisolone Ecrase Duree 6.3 jours
8/07/2006 DEXAMETHASONE Inflation Duree 2 mois
8/07/2006 Methylprednisolone Inflation Duree 2 mois
5/07/2006 Dexamethasone Cream

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : Signature of person tested :	Signature du Médecin : Signature of Doctor :	Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE

USADA 0495

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 26 juillet 2006

28461

RAPPORT D'ANALYSE N° 193/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr TOLLENAERE

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 : 20ème étape à PARIS**
Date :	**23/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**23/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**3 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**2**
Référence de(s) l'échantillon(s) :	**193/07_994171 -**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **24/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N° 193/07

Résultats des rapports T/E

Référence des échantillons	T/E
994171	1.0

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP ROAD CA 92562 MURRIETA (USA)

N° de licence : 0020272

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14 10 1975

Sexe / Sex : M ☒ F ☐

Type de contrôle / Type of test :

Inopiné : Oui ☐ Non ☒

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : UCI - FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 19ème ETAPE

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 22/07/2006

Heure de remise de la notification : 17 H 30

Lieu du contrôle / Place of test : MONTCEAU LES MINES UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : LANDIS
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : MERAVIGLIA GIOVANNI

Nom et prénom du médecin / Name of doctor : (en toutes lettres) BORDABERRY GERARD

Signature du Médecin : Signature of Doctor

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17 H 50

Date du prélèvement / Sampling date : 22/07/2006

Heure du prélèvement / Sampling time : 18 H 05

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994080

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 ml

Densité / Specific Gravity : 1,030

pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine) / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine) :

Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

5.5.06 Dexamethasone Methylprednisolone 6 Oued AUT
Xylocaine / Lidocaine
8.7.06 Dexamethasone injection 2 ml AUT
9.7.06 Methylprednisolone injection AUT
7-5-06 Medrol Tab AUT

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : Bandelettes urinaires Trace protéines

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate : Meraviglia Giovanni

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

6002922 1 IMPRIMERIE NATIONALE

USADA 0498

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 26 juillet 2006

28459

RAPPORT D'ANALYSE N° 188/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BORDABERRY

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 : 19ème étape à Montceau les mines**
Date :	**22/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**22/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**3 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**2**
Référence de(s) l'échantillon(s) :	**188/07_994080**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **23/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B).

Conclusions : **Recherche de substances dopantes négative**

NB : Echantillon N° 994080 : Les résultats des essais ES04 et ES05 ne sont pas totalement exploitables en raison d'une inhibition de la dérivation due à une interférence matricielle.

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N° 188/07

Résultats des rapports T/E

Référence des échantillons	T/E
994080	2.5

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS, FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : U.S.A.

Adresse / Postal adress : 23356 BISHOP ROAD, CA - 92562 MURRIETA, U.S.A

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 00202721 USA 1975 10 14

Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : USA

Date de naissance / Birth Date : 1975/10/14

Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : UCI FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : 2ME TOUR DE FRANCE 15 ET

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 18-07-2006

Heure de remise de la notification : 16.10 h.

Lieu du contrôle / Place of test : L'ALPE D'HUEZ UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested LANDIS, FLOYD

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VAN GESTEL, JAN

Nom et prénom du médecin / Name of doctor : (en toutes lettres) TOLLENAERE, BRUNO

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17.40 h.

Date du prélèvement / Sampling date : 18-07-2006

Heure du prélèvement / Sampling time : 17.50 h.

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994075

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 mL

Densité / Specific Gravity : 1,020

pH / pH : 6.0

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n° | Deuxième n° / Second n° | Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)

5/05/2006 Dexamethasone locale Durée 6 - 3 semaines.
5/05/2006 Methyl prednisone locale Durée 6 à 3 semaines
3/7/2006 Dexamethasone intra articulaire Durée 2 mois.
3/7/2006 Methyl prednisone intra articulaire Durée 2 mois.

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : (AUT)

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE 6002922 1

LABORATOIRE NATIONAL
DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 22 juillet 2006

28384

RAPPORT D'ANALYSE N° 151/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr TOLLENAERE

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 15ème étape à l'Alpe d'Huez**
Date :	**18/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**18/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**2 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**3**
Référence de(s) l'échantillon(s) :	**151/07_994075**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **19/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AUX RAPPORTS D'ANALYSE N° 151/07 et 151/07-00

Résultats des rapports T/E

Référence des échantillons	T/E
994075	1.8

J. de CBAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS, FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : U.S.A.

Adresse / Postal adress : 23356 BISHOP ROAD, CA 92562 MURRIETA, U.S.A.

N° de licence : 00202772 / USA 1975/10/14

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Nationalité / Nationality : U.S.A. — Date de naissance / Birth Date : 1975/10/14 — Sexe / Sex : M ☒ F ☐

Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒

Fédération – Discipline / Sport : UCI FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 12EME ET.

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 14-07-2006 — Heure de remise de la notification : 16.30h — Lieu du contrôle / Place of test : CARCASSONNE UNITÉ MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested : LANDIS, FLOYD

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VAN GESTEL, JAN

Nom et prénom du médecin / Name of doctor : (en toutes lettres) OLIVIER BROCHART

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17.15 h — Date du prélèvement / Sampling date : 14-07-2006 — Heure du prélèvement / Sampling time : 17.25 h.

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B — Code Flacon (et container) n° / bottle Code : 994276

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 cc

Densité / Specific Gravity : 1,030 — pH / pH : 5.5

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non — Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)

5/5/06 Dexamethasone + Methylprednisolone AUT aprés 01/06 (local) 6-8 weeks

8/7/06 Dexamethasone + Methylprednisolone : intra-articular

7/3/06 Prednisolone (cream) AUT depuis 2 mois

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE 6 0022221

LNDD
LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

REÇU le
21 JUIL. 2006

cofrac
ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 18 juillet 2006

28321

RAPPORT D'ANALYSE N° 128/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BROCHART

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 : 12ème étape à CARCASSONNE**
Date :	**14/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**14/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**3 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**1**
Référence de(s) l'échantillon(s) :	**128/07_994276**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **15/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C*, ES04, ES05*) et liquide (ES03, ES03B*) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B*).

Conclusions : Recherche de substances dopantes négative

J. de CEAURRIZ
Directeur

Destinataires :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

** Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°128/07

Résultats des rapports T/E

Référence des échantillons	T/E
994276	2.0

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

Contrôle urinaire / Urine test ☒ — Contrôle sanguin / Blood test ☐

1. En compétition / In competition ☒ Hors compétition / Out of competition ☐
2. Sexe / Sex: Masculin / Male ☒ Féminin / Female ☐
3. Date: 13/07/2006
4. Lieu / Place: PLA-DE-BÉRET
5. Course (nom, étape): TOUR DE FRANCE 11ème ETAPE
 Race (name, stage):
6. Discipline: ROUTE GRAN TOUR
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD
 First name:
 Adresse du coureur: 23356 BISHOP ROAD CA 92562 MURRIETA (USA)
 Address of the rider:
8. Code UCI: USA 19751011
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA
 National federation which delivered the license:
10. Numéro de licence: 0020272
 License number:
11. Tiré au sort / Random: Oui / Yes ☐ Non / No ☒
12. Heure de présentation: 18H00
 Presentation time:
 Volume 100 ml pH* 5
 *Optionnel / Optional
13. Heure de prélèvement: 18H20
 Time of sampling:
 Densité / Specific gravity* 1,030
14. Code des flacons: 994277 A+B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris:
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé:
Contents of the health booklet: 5 5 06 Decanathan + Lacah 6 8 week
05 05 06 Methylprednisolone Lacah 6 8 Week
8 7 06 Decanathan injection 2 mls
8 7 06 Methylprednisolone 2 mls

AVT

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☒ Non / No ☐
 Therapeutic use exemption (TUE)
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Traces Protéines en bandelettes urinaires

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur: SEMIR (nom/name) (signature)
20. Médecin contrôleur: BORDABERRY GERARD (nom/name) (signature)
 Agent de prélèvement sanguin:
 Blood collection officer: (nom/name) (signature)
21. Inspecteur antidopage: MERAVIGLIA GIOVANNI (nom/name) (signature)
 Antidoping inspector:

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 19 juillet 2006

28328

RAPPORT D'ANALYSE N° 121/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BORDABERRY

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 : 11ème étape à Pla-de-Beret (Espagne)**
Date :	**13/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**14/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	
Référence de(s) l'échantillon(s) :	**121/07_994277**

Résultats (Cf référentiels en vigueur : AMA 2006 : Contrôle en compétition)

Date de début des analyses : **15/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B).

Conclusions : **Recherche de substances dopantes négative**

NB : Echantillon N° 994277 : Les résultats de l'essai ES02 ne sont pas totalement exploitables en raison d'une inhibition de la dérivation due à une interférence matricielle.

J. de CEAURRIZ
Directeur

Destinataire :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N° 121/07

Résultats des rapports T/E

Référence des échantillons	T/E
994277	2.5

J. de CHAUDRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP ROAD CA 92562 MURRIETA (USA)

N° de licence : 0020272

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14 10 1975

Sexe / Sex : M ☒ F ☐

Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒

Fédération – Discipline / Sport : UCI FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 9ème ETAPE

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 11/07/2006 — Heure de remise de la notification : 17 10 — Lieu du contrôle / Place of test : DAX (UNITE MOBILE)

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : LANDIS
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : MERAVIGLIA GIOVANNI

Nom et prénom du médecin / Name of doctor : (en toutes lettres) BORDA BERRY GERARD

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17 H 15

Date du prélèvement / Sampling date : 11/07/2006

Heure du prélèvement / Sampling time : 17 H 30

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994203

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 ml

Densité / Specific Gravity : 1,030

pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non — Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)
5 5 06 Dafalgan 1 cp le 6 Juillet
5 5 06 Voltarène 1 cp le 6 Juillet

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : Bandelettes urinaires Tests positifs

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé / Signature of person tested

Signature du Médecin / Signature of Doctor :

Signature de l'accompagnateur (éventuel) / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE 9 0023232 1

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

REÇU le
21 JUIL. 2006

cofrac ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 14 juillet 2006

28276

RAPPORT D'ANALYSE N° 99/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BORDABERRY

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 : 9ème étape à Dax**
Date :	**11/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**11/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**3 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**2**
Référence de(s) l'échantillon(s) :	**99/07_994203**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **12/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C*, ES04, ES05*) et liquide (ES03, ES03B*) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B*).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N° 99/07

Résultats des rapports T/E

Référence des échantillons	T/E
994203	1.3

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

Contrôle urinaire / Urine test ☒ — Contrôle sanguin / Blood test ☐

1. En compétition / In competition ☒ — Hors compétition / Out of competition ☐
2. Sexe / Sex: Masculin / Male ☒ — Féminin / Female ☐
3. Date: 3/07/2006
4. Lieu / Place: ESCH SUR ALZETTE
5. Course (nom, étape) / Race (name, stage): TOUR DE FRANCE 2ème ETAPE
6. Discipline / Discipline: ROUTE G.T.
7. Nom du coureur / Surname of rider: LANDIS — Prénom / First name: FLOYD

Adresse du coureur / Address of the rider: 23356 BISHOP ROAD CA 92562 MURRIETA (USA)

8. Code UCI / UCI Code: USA 19751014
9. Fédération nationale ayant délivré la licence / National federation which delivered the license: USA
10. Numéro de licence / License number: 0020272
11. Tiré au sort / Random: Oui / Yes ☒ Non / No ☐
12. Heure de présentation / Presentation time: 17,25
13. Heure de prélèvement / Time of sampling: 17,30

Volume _____ pH* _____ Densité / Specific gravity* _____

*Optionnel / Optional

14. Code des flacons / Bottle codes: 995462 A+B
15. Heure de refus du coureur / Time of rider's refusal:
16. Médicaments pris / Pharmaceutical drugs taken by rider:

Contenu du livret de santé / Contents of the health booklet:
5 05 06 Decap + Thera + Triamcinolone Locale 6-8 Week AUT
5 05 06 Methyl prednisolone Triamcinolone Acetate 6-8 Week AUT

17. Autorisation d'usage à des fins thérapeutiques (AUT) / Therapeutic use exemption (TUE) — Oui / Yes ☒ Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement / Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

OK

Signature du coureur qui accuse également réception de sa copie / Rider's signature who also acknowledges receipt of his copy: _____

19. Accompagnateur / Assistant: YEMIR (nom/name) _____ (signature)
20. Médecin contrôleur / Examining doctor: BORDABERRY GERARD (nom/name) _____ (signature)

Agent de prélèvement sanguin / Blood collection officer: _____ (nom/name) _____ (signature)

21. Inspecteur antidopage / Antidoping inspector: MERAVIGLIA GIOVANNI (nom/name) Meravigha Giovanni (signature)

LABORATOIRE NATIONAL
DE DEPISTAGE DU DOPAGE

cofrac
ESSAIS
ACCRÉDITATION
N° 1-1174
PORTÉE
COMMUNIQUÉE
SUR DEMANDE

Châtenay-Malabry, le 06 juillet 2006

28132

RAPPORT D'ANALYSE N° 19/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BORDABERRY

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 : 2ème étape à Esch-sur-Alzette**
Date :	**03/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**03/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**1**
Référence de(s) l'échantillon(s) :	**19/07_995462**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **04/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C*, ES04, ES05*) et liquide (ES03, ES03B*) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B*).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

** Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°19/07

Résultats des rapports T/E

Référence des échantillons	T/E
995462	2.8

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP RD MURRIETA (USA)

Organisme demandant le contrôle / Organization requesting the test :
- ☒ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☐ Fédération internationale

N° de licence : 002 02 72

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : U.S.A. Date de naissance / Birth Date : 24.10.1975 Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : PARIS-NICE. RASTEAU (84)

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 23/03/2006 Heure de remise de la notification : 16 h 20 Lieu du contrôle / Place of test : MOBILHOME - ARRIVEE ETAPE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested : LANDIS

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VANDEVYVERE Marc

Nom et prénom du médecin / Name of doctor (en toutes lettres) : DE BROUCKER Bernard

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 16 h 45 Date du prélèvement / Sampling date : 9.3.2006 Heure du prélèvement / Sampling time : 16 h 59

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 874285

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 ML

Identification / Identification :
Licence / Licence : ☒
Carte d'identité / ID : ☐
Passeport / Passport : ☐
Autre / Other : ☐

Densité / Specific Gravity : 1,025 pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal : Oui ☐ Non ☐

Empêchement / Obstacle : Oui ☐ Non ☐

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)
PREDNICARBATE Permande (07/02/06)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : OK

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé / Signature of person tested :

Signature du Médecin : Signature of Doctor :

Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

LNDD
LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

REÇU le
31 MARS 2006

cofrac
ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 27 mars 2006

27437

RAPPORT D'ANALYSE N° 59/03-2

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr PROTHON

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**PARIS/NICE à RASTEAU**
Date :	**09/03/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**14/03/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**2 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**3**
Référence de(s) l'échantillon(s) :	**59/03_874285** -

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **15/03/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C*, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AUX RAPPORTS D'ANALYSE N°59/03-1, 59/03-2 et 59/03-00

Résultats des rapports T/E

Référence des échantillons	T/E
874285	1.5

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 PRIOR ROAD MURRIETA CA 92562 (U.S.A.)

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :
- ☒ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☐ Fédération internationale

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : USA. Date de naissance / Birth Date : 15.10.1975 Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : PARIS NICE 3 Yvelines

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 08 Mars 2006 Heure de remise de la notification : 16 h 10 Lieu du contrôle / Place of test : NICE-ISNE LIGNE D'ARRIVÉE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested Floyd Landis

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VANKEVUYRE MARC

Nom et prénom du médecin / Name of doctor : (en toutes lettres) KHELAIFIA Alain

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 16 h 40 Date du prélèvement / Sampling date : 8 Mars 06 Heure du prélèvement / Sampling time : 16 h 50

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 376013

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 125 ML

Densité / Specific Gravity : 1.020 pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n° | Deuxième n° / Second n° | Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)
PREDNISOLONE le 5 à 10 ? 12 2006
Application locale pour massage

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : OK

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 31 mars 2006

cofrac ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

27472

RAPPORT D'ANALYSE N° 55/03

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr KHENNOUF

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**Paris - Nice à St. ETIENNE**
Date :	**08/03/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**13/03/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	
Référence de(s) l'échantillon(s) :	**55/03_876013**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **14/03/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : **Recherche de substances dopantes négative**

J. de BEAURIE
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°55/03

Résultats des rapports T/E

Référence des échantillons	T/E
876013	1.5

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : Patriot Road 23356 MARIETTA (USA)

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 10207 L

Type de contrôle / Type of test : QUI

Inopiné : Oui ☒ Non ☐

Nationalité / Nationality : U.S.A. Date de naissance / Birth Date : 14.10.1975 Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : UCI Cyclisme

Nom de l'épreuve – Ville – CP / Event and place : Tour Nice (Digne les Bains)

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 10.3.2006 Heure de remise de la notification : 15h20 Lieu du contrôle / Place of test : DIGNE les BAINS

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested LANDIS F.

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VANDEVYVERE Marc

Nom et prénom du médecin / Name of doctor : (en toutes lettres) THEZET Pierre

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 16h00 Date du prélèvement / Sampling date : 10 03 2006 Heure du prélèvement / Sampling time : 16h03

Identification / Identification : Licence / Licence : ☒ Carte d'identité / ID : ☐ Passeport / Passport : ☐ Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : FR2264

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 120 ML

Densité / Specific Gravity : 1,010 pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

ellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)
PRETNACABAT Desmaton (prednisolone) et Cleno BC 7/3/06

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : O.K.

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : Signature of person tested :

Signature du Médecin : Signature of Doctor :

Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) : D. Kleinschmidt

Exemplaire destiné au Président de la Fédération Internationale

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

REÇU le
29 MARS 2006

cofrac ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 24 mars 2006

27413

RAPPORT D'ANALYSE N° 78/03

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr FREZET

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**Paris/Nice à DIGNE LES BAINS**
Date :	**10/03/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**15/03/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**1**
Référence de(s) l'échantillon(s) :	**78/03_872364**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **16/03/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : Recherche de substances dopantes négative

J. de CEAURRIZ
Directeur

Destinataires :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

** Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°78/03

Résultats des rapports T/E

Référence des échantillons	T/E
872364	1.3

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : SANDI FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : GILMORE ROAD 4335 MURRIETTA CA 92562

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 10020572

Type de contrôle / Type of test : Inopiné : Oui ☒ Non ☐

Nationalité / Nationality : USA. Date de naissance / Birth Date : 24.10.1975 Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : CYCLISME - UCI

Nom de l'épreuve – Ville – CP / Event and place : PARIS-NICE CANNES

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 11.3.2006 Heure de remise de la notification : 15h30 Lieu du contrôle / Place of test : CANNES (FRA)

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested : Floyd Landis

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VANDEVUVERE ADEC

Nom et prénom du médecin / Name of doctor : (en toutes lettres) [illegible]

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 16h20 Date du prélèvement / Sampling date : 11.3.2006 Heure du prélèvement / Sampling time : 16h20

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 3715[illegible]

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 ML

Densité / Specific Gravity : 1.030 pH / pH :

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n° | Deuxième n° / Second n° | Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)

Prednisolone (Decortone) 5mg

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

cofrac ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 27 mars 2006

27430

RAPPORT D'ANALYSE N° 75/03

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr CORDONNIER

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**Paris/Nice à CANNES**
Date :	**11/03/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**15/03/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**1**
Référence de(s) l'échantillon(s) :	**75/03_872357**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **16/03/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : Recherche de substances dopantes négative

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète - 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°75/03

Résultats des rapports T/E

Référence des échantillons	T/E
872357	1.6

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : BISMOP ROAD 28356 MURRIETA (U.S.A)

N° de licence : 00202712

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14.10.79

Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : UCI / Cycling - Mountain bike

Nom de l'épreuve – Ville – CP / Event and place : Paris - Nice Nice 06 000

Organisme demandant le contrôle / Organization requesting the test :
- ☒ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☐ Fédération internationale

Type de contrôle / Type of test :

Inopiné : Oui ☐ Non ☒

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 12.03.06

Heure de remise de la notification : 14 h 10'

Lieu du contrôle / Place of test : Pob. Nice - 1 pas démarré

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested : LANDIS F

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VAN DE VUVER ANNE

Nom et prénom du médecin / Name of doctor : (en toutes lettres) Docteur T. Chenet.

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 15 h 25'

Date du prélèvement / Sampling date : 12.03.06

Heure du prélèvement / Sampling time : 15 h 50'

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 7760044

Quantité totale d'urine, ml / Total amount of urine, ml : 125 ml

Densité / Specific Gravity : 1.020

pH / pH : 6

Échantillon insuffisant / Insufficient sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n° | Deuxième n° / Second n° | Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☐ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)
Prednicarbat (Dermatop®) pommade une fois le 7.3.06 sur le peau

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : OK

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE 4 0084101

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

cofrac ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 24 mars 2006

27411

RAPPORT D'ANALYSE N° 66/03-2

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr GARDIOL

Sport : **Cyclisme (UCI)**
Épreuve et lieu : **Paris/Nice à NICE**
Date : **12/03/2006**

Réception de(s) l'échantillon(s) d'urine :

Date : **14/03/2006**
Type de matériel : **Berlinger**
Nombre d'échantillon(s) : **3 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse : **2**
Référence de(s) l'échantillon(s) : **66/03_876004 -**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **15/03/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AUX RAPPORTS D'ANALYSE N°66/03-1 et 66/03-2

Résultats des rapports T/E

Référence des échantillons	T/E
876004	1.6

J. de CÉALURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentiel et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS, FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : U.S.A.

Adresse / Postal adress : 23356 BISHOP ROAD, MURRIERA CALIFORNIA U.S.A.

N° de licence : 002 0272 / USA 19751014

Organisme demandant le contrôle / Organization requesting the test :
- ☒ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Nationalité / Nationality : U.S.A.

Date de naissance / Birth Date : 14.10.1975

Sexe / Sex : M ☒ F ☐

Type de contrôle / Type of test :

Inopiné : Oui ☐ Non ☒

Fédération – Discipline / Sport : F.F. DE CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : T. DE F. 15-IEME ETAPE

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 17-07-2005

Heure de remise de la notification : 16.50 h

Lieu du contrôle / Place of test : L'AUTOBUS CONTR. A.D.

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested LANDIS FLOYD

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : JAN VAN GESTEL

Nom et prénom du médecin / Name of doctor : (en toutes lettres) HUBERT LONE

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17-30 h

Date du prélèvement / Sampling date : 17-07-2005

Heure du prélèvement / Sampling time : 17.40 h

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 874535

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 75 cc

Densité / Specific Gravity : 1,025

pH / pH : 6.0

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

(1.) AUT Betamethasone INJ (12.05.05)

(2.) AUT Betamethasone INJ 08.06.05
1 piqûre dernière / remise

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : Signature of person tested :

Signature du Médecin : Signature of Doctor :

Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 22 juillet 2005

25814

RAPPORT D'ANALYSE N° 85/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr LONG

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2005 (15ème étape) à St Lary Soulan**
Date :	**17/07/2005**

Réception de(s) l'échantillon(s) d'urine :

Date :	**17/07/2005**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**3 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**4**
Référence de(s) l'échantillon(s) :	**85/07_874535**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **18/07/2005**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES01, ES02, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : Recherche de substances dopantes négative

NB1 : Echantillon n° 874535 : Les résultats des essais ES04 et ES05 ne sont pas totalement exploitable en raison d'une inhibition de la dérivation due à une interférence matricielle.

J. de CHAVARIZ
Directeur

Destinataires :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°85/07 et 85/07-00

Résultats des rapports T/E

Référence des échantillons	T/E
874535	2.2

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

Johncie Wingard

From: Varin Christian - UCI [christian.varin@uci.ch]
Sent: Thursday, August 10, 2006 6:39 AM
To: Travis Tygart
Cc: Lautenschlager Delphine - UCI
Subject: Floyd Landis request

Dear Travis,

Floyd Landis requested (through his team doctor) all his results (urine and blood anti-doping tests) for 2005 – 2006.

Would USADA agree that we send these results to Floyd Landis team doctor? Or does USADA prefer that we direct the rider to USADA?

Thank you for your help in this matter!

Yours sincerely,

Christian Varin
Manager Antidopage / Antidoping Manager
Union Cycliste Internationale
CH - 1860 Aigle

Tél : +41-24.468.58.11
Fax : +41-24.468.58.12
w[illegible]uci.ch

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

Linda Barnes

From: Ana Reyes [afreyes@ucla.edu]
Sent: Thursday, August 17, 2006 2:09 PM
To: Linda Barnes
Subject: RE: Request for t/e ratio info

Hi Linda,

We'll be glad to provide you with the info provided USADA sends us a written ok from UCI to release T/E to you.

Thanks,
Ana

Ana Reyes
Service/Quality Manager
UCLA Olympic Analytical Laboratory
Main Phone: (310) 825-2635
FAX: (310) 206-9077
E-mail:afreyes@ucla.edu

From: Linda Barnes [mailto:lbarnes@usantidoping.org]
Sent: Wednesday, August 16, 2006 3:16 PM
To: Ana Reyes
Subject: Request for t/e ratio info

Ana ---

ure if you can help with this, but we are in the process of reviewing an athlete's T/E ratio history. We have received samples #'s that were collected by UCI and analyzed at the UCLA laboratory. Would it be possible for us to get the T/E ratio on these samples?

Please let me know –

Thanks for your extra work!

Linda Barnes
Testing Results Manager
719-785-2024
lbarnes@usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

Sample Collected by UCI - Analyzed by UCLA

Date	Sample #
4/21/2005	920460
4/22/2005	920462
2/22/2006	951826
2/23/2006	951875
2/24/2006	951828
2/25/2006	951866
2/26/2006	951872
4/20/2006	951792
4/21/2006	951786
4/22/2006	951787
4/23/2006	951790

Johncie Wingard

From: Johncie Wingard on behalf of Travis Tygart
Sent: Thursday, September 07, 2006 8:46 AM
To: 'Lautenschlager Delphine - UCI'
Subject: RE: steroid profiles

Ms. Lautenschlager – I am responding on Travis Tygart's behalf as he is out of the office today. Yes, we did receive the steroid profiles directly from the Châtenay-Malabry laboratory on the rider's other Tour samples. Could you please also provide us with the same information including Doping Control form and steroid profile data from the laboratories, for **all** prior samples collected by UCI for this rider? We are gathering the same information from the samples that we have collected.

Also, it might be helpful if UCI would grant us authority to contact each of the labs directly about these specific samples if necessary. Please let us know if UCI would consent to this.

Thank you for your assistance in this matter.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

From: Lautenschlager Delphine - UCI [mailto:Delphine.Lautenschlager@uci.ch]
Sent: Thursday, September 07, 2006 2:47 AM
To: Travis Tygart
Subject: steroid profiles

Dear Travis,

We received the « steroid profiles » from the laboratory of Châtenay-Malabry for the file 29/06.

Have you received a copy too? Or do you want that we send you a copy of this document?

We are looking forward to hearing from you.

Best regards,

Delphine Lautenschlager
Assistante
Service Antidopage
Antidoping Services
Union Cycliste Internationale
CH - 1860 Aigle

Tél : +41-24.468.58.11
Fax : +41-24.468.58.12
www.uci.ch

IMPORTANT NOTICE :

This message contains confidenti. formation and is intended only for the ind. ual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

Johncie Wingard

From: Travis Tygart
Sent: Tuesday, September 12, 2006 7:41 AM
To: Johncie Wingard
Subject: FW: file 29/06

Can you send me your email?

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Lautenschlager Delphine - UCI [mailto:Delphine.Lautenschlager@uci.ch]
Sent: Tuesday, September 12, 2006 3:00 AM
To: Travis Tygart
Subject: file 29/06

Dear Travis,

We refer to the e-mail of Mr. Johncie B. Wingard, in which he asks us the authorisation to contact directly each of the labs about the specific samples.

We need to know whether you need the doping control forms and labs results or if you already have them.

If you are already in possession of these documents, the UCI agrees that USADA contact each of the labs directly to get the steroid profiles.

In more, we kindly ask you to provide us with the defence of the rider in which he contests the reliability of the laboratory.

We are looking forward to hearing from you.

Best regards,

Delphine Lautenschlager
Assistante
Service Antidopage
Antidoping Services
Union Cycliste Internationale

CH - 1860 Aigle

Tél : +41-24.468.58.11
Fax : +41-24.468.58.12
www.uci.ch

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

Johncie Wingard

From: Johncie Wingard
Sent: Tuesday, September 12, 2006 8:24 AM
To: 'Lautenschlager Delphine - UCI'
Subject: file 29/06

Ms. Lautenschlager – Travis asked me to follow up with you after he talked to you this morning. Christian Varin provided us with some documents for this rider in correspondence dated 9 August, 2006. Those documents included a chart of all of the samples collected by UCI for this rider including sample number, date, event and T/E ratio if available. We received the Doping Control Official Record and lab test results for the following samples:

874535
876013
874285
872364
872357
876004
995462
994203
994277
994276
994075
994080
994171

We would appreciate receiving the Doping Control Official Record and laboratory test results for all of the other samples listed in the chart which would be for all the samples collected from 1999 through 2004. If you could please provide those documents to us, we will contact the laboratories directly for the steroid profile data.

We greatly appreciate your assistance in this matter. If you have any questions, please do not hesitate to contact Travis directly.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

Johncie Wingard

From: Johncie Wingard
Sent: Tuesday, September 12, 2006 10:08 AM
To: 'Lautenschlager Delphine - UCI'
Subject: File 29/06

Ms. Lautenschlager – after closer review of the documents sent by Mr. Varin, besides all of the samples from 1999-2004, we also still need Doping Control Official Records and laboratory reports for several samples in 2005 & 2006 as listed below:

920460
920462
951826
951875
951828
951866
951872
951792
951789
951787
951790

I apologize for omitting these from my earlier message.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

Johncie Wingard

ɔm: USADA Spam
Sent: Wednesday, September 13, 2006 2:25 PM
To: Travis Tygart
Subject: FW: ttygart@usantidoping.org - Message is from an unknown sender - Floyd Landis case
Importance: High

To prevent this e-mail from going thru the spam filter reply to the senders e-mail address or let the IT Department know and we will exclude from the spam filter for you.

Thank You,

Spam Filter

From: Peruzzi Erica - UCI [mailto:erica.peruzzi@uci.ch]
Sent: Wednesday, September 13, 2006 12:47 AM
To: Travis Tygart
Subject: ttygart@usantidoping.org - Message is from an unknown sender - Floyd Landis case
Importance: High

Good morning Travis,

As promised, I am pleased to tell you that I spoke with Me Verbiest regarding Floyd's blood results.
He confirmed that Floyd Landis received all his blood results directly from him and therefore can provide you all the papers you need.
We may not send you any result or value without his agreement, which is the normal procedure for us.

Would you be kind to contact Landis about his results? He should be able to provide them to you.

Hoping that I could give you some more explanation I remain,

Kind Regards,

Erica

Erica Peruzzi
Assistante du Service Santé
Assistant Health Services
Union Cycliste Internationale
CH - 1860 Aigle

Tél : +41-24.468.58.11
Fax : +41-24.468.58.12
www.uci.ch

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

Johncie Wingard

From: Travis Tygart
Sent: Tuesday, September 19, 2006 4:36 PM
To: Paul Scott
Cc: Johncie Wingard; Travis Tygart
Subject: UCLA request
Importance: High
Attachments: Ltr FROM UCLA to USADA.doc; UCLA Chart .xls

Dear Paul-

I am requesting steroid profile data concerning the samples on the attached letter. All of these samples were analyzed by the UCLA laboratory.

I have included an "Example" of a response letter as a guide to your laboratory to make this request easier for you. I have also included a chart that has the category for the steroid profile. If you do not have the data concerning a particular sample or steroid, please indicate with an "N/A" or similar notation.

If you have any questions or concerns, please feel free to contact me directly.

Sincerely,

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Johncie Wingard
Sent: Tuesday, September 19, 2006 2:16 PM
To: Travis Tygart
Subject: UCLA request

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency

1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

EXAMPLE

[Date]

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: Sample #s: 920460, 920462, 951826, 951875, 951828, 951866, 951872, 951792, 951789, 951787, 951790

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples are accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Don Catlin
Director

Enclosure

URINE RESULTS FROM WADA ACCREDITED LABS
Floyd Landis

Bottle#	Collection	SG	TE	[Test]	[Epit]	[Andr]	[Etio]	[5aAD]	5bAD]	DHEA
920460	04/21/05									
920462	04/22/05									
951826	02/22/06									
951875	02/23/06									
951828	02/24/06									
951866	02/25/06									
951872	02/26/06									
951792	04/20/06									
951789	04/21/06									
951787	04/22/06									
951790	04/23/06									

Johncie Wingard

From: Linda Barnes
Sent: Tuesday, September 19, 2006 8:20 AM
o: 'Lautenschlager Delphine - UCI'
Cc: Johncie Wingard; Travis Tygart
Subject: RE: file 29/26

Attachments: Athlete Response.pdf; Athlete Response - Exhibits.pdf


Athlete esponse.pdf (453 KI


Athlete Response - Exhibits.pd...

Attached is the athlete's response that you requested.

Linda Barnes
Testing Results Manager
719-785-2024
lbarnes@usantidoping.org

-----Original Message-----
From: Travis Tygart
Sent: Tuesday, September 19, 2006 7:39 AM
To: Lautenschlager Delphine - UCI
Cc: Johncie Wingard; Linda Barnes
Subject: RE: file 29/26

Delphine-

I apologize for not forwarding you the athlete's submission. I did not receive your request. Given that this is a UCI test, we will happily send you the submission. We will also copy you on the recommendation from the Anti-Doping Review Board.

I will send the documents under separate cover.

Sincerely,

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel United States Anti-Doping Agency 1330 Quail Lake Loop, Suite 260 Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028

Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally

privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Lautenschlager Delphine - UCI [mailto:Delphine.Lautenschlager@uci.ch]
Sent: Tuesday, September 19, 2006 7:11 AM
To: Travis Tygart
Subject: file 29/26

Dear Travis,

We acknowledge receipt of your request sent by Mr. Johncie Wingard. It is still in process.

On 12th September, I send you a request by e-mail:

"We kindly ask you to provide us with the defence of the rider in which he contests the reliability of the laboratory"

Would you be kind enough to send us this document as soon as possible?

We thank you in advance and are looking forward to hearing from you.

Best regards,

Delphine Lautenschlager

Assistante

Service Antidopage

Antidoping Services

Union Cycliste Internationale

CH - 1860 Aigle

Tél : +41-24.468.58.11

Fax : +41-24.468.58.12

www.uci.ch <http://www.uci.ch/>

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

Johncie Wingard

From: Travis Tygart
Sent: Wednesday, September 20, 2006 11:51 AM
To: agustinf.rodriguez@csd.mec.es
Cc: Travis Tygart; Johncie Wingard
Subject: Request for Laboratory Data.
Importance: High
Attachments: Ltr FROM Madrid to USADA.doc; Madrid Chart .xls

Dear Dr. Cano-

I hope this email finds you well.

I am writing to request steroid profile data concerning the samples on the attached letter. All of these samples were analyzed by the UCLA laboratory.

I have included an "Example" of a response letter as a guide to your laboratory to make this request easier for you. I have also included a chart that has the category for the steroid profile. If you do not have the data concerning a particular sample or steroid, please indicate with an "N/A" or similar notation.

If you have any questions or concerns, please feel free to contact me directly.

Thank you for your assistance with this important matter.

Sincerely,

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Johncie Wingard
Sent: Wednesday, September 20, 2006 11:34 AM
To: Travis Tygart
Subject:

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

EXAMPLE

[Date]

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: UCI Sample #s: 311370, 336873, 342599, 342856, 342850, 348369, 347883

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples are accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Agustin-Francisco Rodriguez Cano
Director

Enclosure

URINE RESU FROM MADRID LAB
Male Cycling Athlete

Bottle#	Collection	SG	TE	[Test]	[Epit]	[Andr]	[Etio]	[5aAD]	5bAD]	DHEA
311370	04/30/00		2.10							
336873	08/15/03		1.50							
342599	09/04/04		1.70							
342856	09/11/04		2.00							
342850	09/12/04		1.40							
348369	09/13/04		1.30							
347883	09/14/04		1.00							

USADA 0555

*** TX REPORT ***

TRANSMISSION OK

TX/RX NO	4772
RECIPIENT ADDRESS	901134915437290
DESTINATION ID	
ST. TIME	09/25 12:01
TIME USE	01'07
PAGES SENT	4
RESULT	OK

USADA™
U.S. Anti-Doping Agency
Integrity. Health. Sport.

Facsimile Cover Sheet

To:	Dr. Agustin- Francisco Rodriguez Cano
Company:	
Phone:	
Fax:	34 91 543 72 90
From:	**Travis T. Tygart**
Company:	**U.S. Anti-Doping Agency**
Address:	1330 Quail Lake Loop, Suite 260 Colorado Springs, Colorado 80906-4651 USA
Direct:	+719-785-2031
Fax:	+719-785-2001
e-mail:	
Date:	9/25/2006
Pages including this cover page:	4

Comments:

Please find attached a copy of the email I previously sent to you. I look forward to your resonse. Thank you.

Johncie Wingard

m: Travis Tygart
Sent: Wednesday, September 20, 2006 11:48 AM
To: direction@indd.com
Cc: Johncie Wingard; Travis Tygart
Subject: Request for Laboratory Data.
Importance: High
Attachments: Paris Chart .xls; Ltr FROM Paris to USADA.doc

Dear Professor Ceaurriz-

I hope you are doing well.

I am requesting steroid profile data concerning the samples on the attached letter. All of these samples were analyzed by the Paris laboratory.

I have included an "Example" of a response letter as a guide for your response. I hope this makes our request as easier for you. I have also included a "Chart" that has the categories for the steroid data we are interested in obtaining. If you do not have the data concerning a particular sample or steroid, please indicate with an "N/A" or similar notation.

If you have any questions or concerns, please feel free to contact me directly.

Thank you for your assistance with this important matter.

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Johncie Wingard
Sent: Wednesday, September 20, 2006 11:33 AM
To: Travis Tygart
Subject:

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

URINE RESUL FROM PARIS LAB
Male Cycling Athlete

Bottle#	Collection	ph	SG	TE	[Test]	[Epit]	[Andr]	[Etio]	[5aAD]	5bAD]	DHEA
186671	36411			2							
185105	36421			2.1							
217241	36762			1.5							
217243	36764			0.9							
216923	36982			1.1							
210038	37068			1.1							
176593	37423			1.2							
808345	37828			0.9							
289130	38055			0.9							
874535	38550			2.2							
876013	38784			1.5							
874285	38785			1.5							
872364	38786			1.3							
872357	38787			1.6							
876004	38788			1.6							

USADA 0559

EXAMPLE

[Date]

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: UCI Sample #s: 186671, 185105, 217241, 217243, 216923, 210038, 176593, 808345, 289130, 874535, 876013, 874285, 872364, 872357, 876004

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples are accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Jacques de Ceaurriz
Director

Enclosure

*** TX REPORT ***

TRANSMISSION OK

TX/RX NO	4773
RECIPIENT ADDRESS	901133146603017
DESTINATION ID	
ST. TIME	09/25 12:03
TIME USE	01'24
PAGES SENT	5
RESULT	OK

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

Facsimile Cover Sheet

To: Pr. Jacques de Ceaurriz
Company:
Phone:
Fax: 33 1 46 60 30 17

From: Travis T. Tygart
Company: U.S. Anti-Doping Agency
Address: 1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906-4651 USA
Direct: +719-785-2031
Fax: +719-785-2001
e-mail:
Date: 9/25/2006
Pages including this cover page: 5

Comments:

Please find attached a copy of the email I previously sent to you. I look forward to your resonse. Thank you.

Johncie Wingard

›m: Paul Scott [pascott@ucla.edu]
Sent: Thursday, September 21, 2006 6:19 PM
To: Travis Tygart
Subject: Steroid Profile Report
Attachments: Steroid Profile Report 9-21-06.pdf

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

UNIVERSITY OF CALIFORNIA, LOS ANGELES

UCLA

BERKELEY • DAVIS • IRVINE • LOS ANGELES • MERCED • RIVERSIDE • SAN DIEGO • SAN FRANCISCO

SANTA BARBARA • SANTA CRUZ

UCLA OLYMPIC ANALYTICAL LABORATORY
DEPARTMENT OF PHARMACOLOGY
UCLA SCHOOL OF MEDICINE
2122 GRANVILLE AVENUE
LOS ANGELES, CALIFORNIA 90025
PHONE: 310-825-2635
FAX: 310-206-9077

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: Sample #s: 920460, 920462, 951826, 951875, 951828, 951866, 951872, 951792, 951789, 951787, 951790

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples have been verified and accurately reflect the values we have recorded in our original records for the samples.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Don H. Catlin, M.D.
Director

Enclosure

URINE RESULTS FROM WADA ACCREDITED LABS

Bottle#	Collection	SG	TE	[Test]	[Epit]	[Andr]	[Etio]	[5aAD]	[5bAD]
920460	04/21/05	1.015	1.5	11	7	710	697	9	32
920462	04/22/05	1.025	1.2	24	21	1416	1491	30	110
951826	02/22/06	1.026	1.8	28	16	1006	1148	18	86
951875	02/23/06	1.022	1.5	29	19	1222	1283	20	71
951828	02/24/06	1.025	1.5	29	20	796	1977	26	137
951866	02/25/06	1.025	1.7	35	22	1621	2079	38	205
951872	02/26/06	1.016	1.8	20	12	1106	1359	14	85
951792	04/20/06	1.025	2.4	44	25	1813	2335	24	182
951789	04/21/06	1.028	1.8	52	34	2425	3018	37	139
951787	04/22/06	1.025	1.5	56	43	1781	2308	60	353
951790	04/23/06	1.025	1.4	68	56	845	924	28	161

Johncie Wingard

From: Travis Tygart
Sent: Monday, September 25, 2006 10:15 AM
To: aishah@dcc.usm.my
Cc: Travis Tygart; Johncie Wingard
Subject: USADA Request for Steroid Profile Data.
Importance: High
Attachments: Penang Chart .xls; Ltr FROM Penang to USADA.doc

Dear Dr. Aishah Latiff-

I hope this email finds you well.

I am writing to request steroid profile data concerning the samples on the attached letter. All of these samples were analyzed by the UCLA laboratory.

I have included an "Example" of a response letter as a guide to your laboratory to make this request easier for you. I have also included a chart that has the category for the steroid profile. If you do not have the data concerning a particular sample or steroid, please indicate with an "N/A" or similar notation.

We would prefer that you provide both the uncorrected and the corrected data. But, it is essential that you provide the uncorrected and the (Ph, SG) so that we can ensure we have all the necessary data as we compare to other laboratory results that we are compiling.

If you have any questions or concerns, please feel free to contact me directly.

Thank you for your assistance with this important matter.

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Johncie Wingard
Sent: Monday, September 25, 2006 9:50 AM
To: Travis Tygart
Subject:

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

EXAMPLE

[Date]

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: UCI Sample #: 055383

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced sample. Additionally, I have reviewed the files related to this sample and hereby confirm the following:

1. The sample was accompanied by appropriate transport chain of custody documentation when it was received at our laboratory.

2. The sample was properly sealed when it was received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the sample while it was in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing the sample.

5. The screen results for the sample is accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Aishah A. Latiff
Director

Enclosure

URINE RESULTS FROM PENANG LAB
Male Cycling Athlete

Bottle#	Collection	SG	TE	[Test]	[Epit]	[Andr]	[Etio]	[5aAD]	5bAD]	DHEA
055383	January 26, 2000									

*** TX REPORT ***

TRANSMISSION OK

TX/RX NO	4775
RECIPIENT ADDRESS	90116046569869
DESTINATION ID	
ST. TIME	09/25 12:07
TIME USE	01'20
PAGES SENT	4
RESULT	OK

USADA™
U.S. Anti-Doping Agency
Integrity. Health. Sport.

Facsimile Cover Sheet

To: Dr. Aishah Latiff
Company:
Phone:
Fax: 60 4 656 98 69

From: Travis T. Tygart
Company: U.S. Anti-Doping Agency
Address: 1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906-4651 USA
Direct: +719-785-2031
Fax: +719-785-2001
e-mail:
Date: 9/25/2006
Pages including this cover page: 4

Comments:

Please find attached a copy of the email I previously sent to you. I look forward to your resonse. Thank you.

Johncie Wingard

From: Travis Tygart
Sent: Monday, September 25, 2006 10:07 AM
To: direction@indd.com
Cc: Johncie Wingard
Subject: Updated Chart for my previous email.
Attachments: Paris Chart .xls

I have updated the chart to make the dates clearer for your use.

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Johncie Wingard
Sent: Monday, September 25, 2006 9:49 AM
To: Travis Tygart
Subject:

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

Johncie Wingard

From: Travis Tygart
Sent: Monday, September 25, 2006 8:39 AM
To: direction@indd.com
Cc: Johncie Wingard
Subject: USADA Request.

I previously sent an email that I hope you will receive although i have had a difficult time getting it to go through to you. I will send it by fax if it is returned again.

In the request, I am asking for steroid profiles and other data from several collections provided by the same athlete. Please provide the raw data so that we can calculate the "corrected values" and show both. You are welcome to provide both but we are asking for the uncorrected but also the data (Ph, SG) that will allow us to corrected it for comparison to other data we are compiling.

Thank you for your assistance.

Sincerely,

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

Johncie Wingard

From: Travis Tygart
Sent: Wednesday, September 20, 2006 11:48 AM
To: direction@indd.com
Cc: Johncie Wingard; Travis Tygart
Subject: Request for Laboratory Data.
Importance: High
Attachments: Paris Chart .xls; Ltr FROM Paris to USADA.doc

Dear Professor Ceaurriz-

I hope you are doing well.

I am requesting steroid profile data concerning the samples on the attached letter. All of these samples were analyzed by the Paris laboratory.

I have included an "Example" of a response letter as a guide for your response. I hope this makes our request as easier for you. I have also included a "Chart" that has the categories for the steroid data we are interested in obtaining. If you do not have the data concerning a particular sample or steroid, please indicate with an "N/A" or similar notation.

If you have any questions or concerns, please feel free to contact me directly.

Thank you for your assistance with this important matter.

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Johncie Wingard
Sent: Wednesday, September 20, 2006 11:33 AM
To: Travis Tygart
Subject:

Johncie B. Wingard

9/20/2006

Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

EXAMPLE

[Date]

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: UCI Sample #s: 186671, 185105, 217241, 217243, 216923, 210038, 176593, 808345, 289130, 874535, 876013, 874285, 872364, 872357, 876004

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples are accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Jacques de Ceaurriz
Director

Enclosure

URINE RESUL FROM PARIS LAB
Male Cycling Athlete

Bottle#	Collection	ph	SG	TE	[Test]	[Epit]	[Andr]	[Etio]	[5aAD]	5bAD]	DHEA
186671	8-Sep-99			2							
185105	18-Sep-99			2.1							
217241	24-Aug-00			1.5							
217243	26-Aug-00			0.9							
216923	1-Apr-01			1.1							
210038	26-Jun-01			1.1							
176593	16-Jun-02			1.2							
808345	26-Jul-03			0.9							
289130	9-Mar-04			0.9							
874535	17-Jul-05			2.2							
876013	8-Mar-06			1.5							
874285	9-Mar-06			1.5							
872364	10-Mar-06			1.3							
872357	11-Mar-06			1.6							
876004	12-Mar-06			1.6							

Johncie Wingard

From: Travis Tygart
Sent: Monday, September 25, 2006 10:12 AM
To: lad@idesporto.pt
Cc: Johncie Wingard; Travis Tygart
Subject: Request for Steroid Profile Data.
Importance: High
Attachments: Ltr FROM Lisbon to USADA.doc; Lisbon Chart .xls

Dear professor Xavier de la Torre-

I hope this email finds you well.

I am writing to request steroid profile data concerning the samples on the attached letter. All of these samples were analyzed by the UCLA laboratory.

I have included an "Example" of a response letter as a guide to your laboratory to make this request easier for you. I have also included a chart that has the category for the steroid profile. If you do not have the data concerning a particular sample or steroid, please indicate with an "N/A" or similar notation.

We would prefer that you provide both the uncorrected and the corrected data. But, it is essential that you provide the uncorrected and the (Ph, SG) so that we can ensure we have all the necessary data as we compare to other laboratory results that we are compiling.

If you have any questions or concerns, please feel free to contact me directly.

Thank you for your assistance with this important matter.

Travis T. Tygart, Esq.
Senior Managing Director, General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906
(T) 719-785-2031
(F) 719-785-2028
Toll Free: 866-601-263
Email: TTygart@usantidoping.org
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it, may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

From: Johncie Wingard
Sent: Monday, September 25, 2006 9:50 AM
To: Travis Tygart
Subject:

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

EXAMPLE

[Date]

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: UCI Sample #: 277821

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced sample. Additionally, I have reviewed the files related to this sample and hereby confirm the following:

1. The sample was accompanied by appropriate transport chain of custody documentation when it was received at our laboratory.

2. The sample was properly sealed when it was received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the sample while it was in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing the sample.

5. The screen results for the sample is accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Xavier de la Torre
Director

Enclosure

URINE RESUL ROM LISBON LAB
Male Cycling Athlete

Bottle#	Collection	SG	TE	[Test]	[Epit]	[Andr]	[Etio]	[5aAD]	5bAD]	DHEA
277821	22-Feb-04									

USADA 0579

*** TX REPORT ***

TRANSMISSION OK

TX/RX NO	4774
RECIPIENT ADDRESS	9011351217977529
DESTINATION ID	
ST. TIME	09/25 12:05
TIME USE	01'07
PAGES SENT	4
RESULT	OK

USADA™
U.S. Anti-Doping Agency
Integrity. Health. Sport.

Facsimile Cover Sheet

To: Prof. Xavier de la Torre
Company:
Phone:
Fax: 351 21 797 75 29

From: Travis T. Tygart
Company: U.S. Anti-Doping Agency
Address: 1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906-4651 USA
Direct: +719-785-2031
Fax: +719-785-2001
e-mail:

Date: 9/25/2006
Pages including this cover page: 4

Comments:

Please find attached a copy of the email I previously sent to you. I look forward to your resonse. Thank you.

LABORATOIRE NATIONAL
DE DEPISTAGE DU DOPAGE

At Châtenay-Malabry, the 29th September 2006

Travis T. TYGART
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs CO 80906
USA

Re : UCI samples n° : 186671, 185105, 217241, 217243
216923, 210038, 176593, 808345
289130, 874535, 876013, 874285
872364, 872357, 876004

Dear Mr. Tygart,

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples are accurate except for sample 874535.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Jacques de CEAURRIZ
Director

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.cor

URINE RESULTS FROM PARIS LAB
Male Cycling Athlete

Bottle	Collection	pH	SG	TE	[Test]	[Epit]	[Andro]	[Etio]	[5αAD]	[5βAD]	DHEA
186671	8 Sept 99	NA	NA	2	NA	NA	NA	NA	NA	NA	NA
185105	18 Sept 99	NA	NA	2.1	NA	NA	NA	NA	NA	NA	NA
217241	24 Aug 00	NA	NA	1.5	NA	NA	NA	NA	NA	NA	NA
217243	26 Aug 00	NA	NA	0.9	NA	NA	NA	NA	NA	NA	NA
216923	1 April 01	NA	NA	1.1	NA	NA	NA	NA	NA	NA	NA
210038	26 Jun 01	6	1.028	1.1	NA	NA	NA	NA	NA	NA	NA
176593	16 Jun 02	6	1.023	1.3	28	21	1502	1515	27	184	36
808345	26 Jul 03	5	1.018	1.3	22	18	848	1267	18	109	17
289130	9 Mar 04	5	1.018	1.3	22	19	1429	1436	25	88	35
* 874535	17 Jul 05	5	1.036	2.2	32	15	570	576	22	41	15
876013	8 Mar 06	5	1.027	1.5	65	46	2302	2755	39	202	50
874285	9 Mar 06	5	1.022	1.5	37	27	1305	1362	26	129	27
872364	10 Mar 06	5	1.022	1.3	39	31	1302	1696	28	167	27
872357	11 Mar 06	5	1.024	1.6	61	41	1905	2509	55	308	49
876004	12 Mar 06	5	1.016	1.6	36	24	1298	1385	28	152	28

Data are concentrations uncorrected for specific gravity : ng/mL
* Analysis of the sample was distorted due to the presence of an inhibition of the derivatization step
NA : Not available

USADA 0582

3rd october, 2006

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: UCI Sample #s: 311370, 336873, 342599, 342856, 342850, 348369, 347883

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples are accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Agustin-Francisco Rodriguez Cano
Director

Enclosure

URINE RESULTS FROM MADRID LAB
Male Cycling Athlete

Bottle#	Collection	SG	TE	[Test]	[Epit]	[Andr]	[Etio]	[5aAD]	5bAD]	DHEA
311370	04/30/00	N/A	2.10	N/A	N/A	N/A	N/A	N/A	N/A	N/A
336873	08/15/03	1.007	1.50	84.1	57.3	4111	2951	73.2	392.0	30.00
342599	09/04/04	1.019	1.70	25.8	14.5	1761	1902	27.9	143.9	21.60
342856	09/11/04	1.014	2.00	7.5	3.7	395	438	5.8	18.4	11.60
342850	09/12/04	1.025	1.40	39.9	29.4	2605	2702	32.4	144.9	27.40
348369	09/13/04	1.019	1.30	25.5	19.4	1646	1301	22.3	153.1	13.40
347883	09/14/04	1.019	1.00	36.2	37.9	2529	1991	32.7	288.7	21.90

USADA 0584

Johncie Wingard

From: Johncie Wingard
Sent: Thursday, October 05, 2006 11:26 AM
To: 'Lautenschlager Delphine - UCI'
Subject: USADA Request

Ms. Lautenschlager – I am following up on the emails that I sent to you on Sept. 12, 2006. I have included the language from those emails below for your convenience. Could you please update me on the status of our requests? We greatly appreciate your assistance in this matter. Thank you.

Ms. Lautenschlager – Travis asked me to follow up with you after he talked to you this morning. Christian Varin provided us with some documents for this rider in correspondence dated 9 August, 2006. Those documents included a chart of all of the samples collected by UCI for this rider including sample number, date, event and T/E ratio if available. We received the Doping Control Official Record and lab test results for the following samples:

874535
876013
874285
872364
872357
876004
995462
994203
994277
994276
994075
994080
994171

We would appreciate receiving the Doping Control Official Record and laboratory test results for all of the other samples listed in the chart which would be for all the samples collected from 1999 through 2004. If you could please provide those documents to us, we will contact the laboratories directly for the steroid profile data.

We greatly appreciate your assistance in this matter. If you have any questions, please do not hesitate to contact Travis directly.

Ms. Lautenschlager – after closer review of the documents sent by Mr. Varin, besides all of the samples from 1999-2004, we also still need Doping Control Official Records and laboratory reports for several samples in 2005 & 2006 as listed below:

920460
920462
951826
951875
951828
951866
951872
951792
951789
951787
951790

apologize for omitting these from my earlier message.

hncie B. Wingard
alegal
ed States Anti-Doping Agency

2006

1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

Presidência do Conselho de Ministros
Secretaria de Estado da Juventude e do Desporto

Instituto do Desporto de Portugal

FAX

REFERÊNCIA: (REFERENCE)	271 /L.A.D./2006
DATA: (DATE)	12-10-06

PARA: (TO)	TRAVIS T. TYGART		
A/C.	USADA	FAX:	+ 1 719 785 2001
DE: (FROM)	INSTITUTO DO DESPORTO DE PORTUGAL – LABORATÓRIO DE ANÁLISES E DOPAGEM		
N.º PÁGINAS: (NUMBER OF PAGES)	2+1		
ASSUNTO:	STEROID PROFILE SAMPLE 277821		

Dear Sirs,

Please find enclosed the documentation regarding sample UCI 277821 requested on your fax dated September 25th 2006.

Do not hesitate to contact us should you have any further question or doubt.

Sincerely

Dr. Xavier de la Torre

Scientific Director

IOP – Modelo nº 1

Presidência do Conselho de Ministros
Secretaria de Estado da Juventude e do Desporto

Instituto do Desporto de Portugal
Laboratório de Análises e Dopagem

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: UCI Sample #: 277821

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced sample. Additionally, I have reviewed the files related to this sample and hereby confirm the following:

1. The sample was accompanied by appropriate transport chain of custody documentation when it was received at our laboratory.

2. The sample was properly sealed when it was received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the sample while it was in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing the sample.

5. The screen results for the sample is accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Xavier de la Torre
Scientific Director

Lisbon, 12th October 2006

IP – Modelo nº 1

URINE RESULTS FROM LISBON LAB
Male Cycling Athlete

Bottle#	Collection	SG	pH	TE	[Test]	[Epit]	[Andr]	[Etio]	[5aAD]	5bAD]	DHEA
277821	22-Feb-04	1,011	5,8	1,00	10,8	10,4	488	415	10,4	21,5	n.a.
		Corrected values			19,6	18,9	887,3	754,5	18,9	39,1	

12/10 2006 10:22 FAX 217935949 LAD 003

USADA 0589

Johncie Wingard

From: Johncie Wingard
Sent: Thursday, October 12, 2006 9:57 AM
To: 'Don (MD) Catlin (dcatlin@ucla.edu)'; 'areyes@ucla.edu'
Subject: FW: Steroid Profile Data
Attachments: Ltr FROM UCLA to USADA 2.doc

Ana – please see our request for steroid profile data below. Thank you.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

From: Johncie Wingard
Sent: Wednesday, October 11, 2006 3:01 PM
To: 'pascott@ucla.edu'
Subject: Steroid Profile Data

Hi Paul – Travis asked me to request steroid profile data on two more samples. The results of these two samples were just reported to Linda Barnes earlier this week. They were follow-up samples on the same athlete for whom you provided steroid data on previously. Attached is a sample letter indicating the two sample numbers. We would appreciate receiving the data as soon as possible. Thanks very much for your assistance and if you have any questions, please contact Travis directly.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

Johncie Wingard

From: Johncie Wingard
Sent: Wednesday, October 11, 2006 3:01 PM
To: 'pascott@ucla.edu'
Subject: Steroid Profile Data
Attachments: Ltr FROM UCLA to USADA 2.doc

Hi Paul – Travis asked me to request steroid profile data on two more samples. The results of these two samples were just reported to Linda Barnes earlier this week. They were follow-up samples on the same athlete for whom you provided steroid data on previously. Attached is a sample letter indicating the two sample numbers. We would appreciate receiving the data as soon as possible. Thanks very much for your assistance and if you have any questions, please contact Travis directly.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

EXAMPLE

[Date]

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: Sample #s: 1501850 and 497104

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples are accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Don Catlin
Director

Enclosure

Johncie Wingard

From: Lautenschlager Delphine - UCI [Delphine.Lautenschlager@uci.ch]
Sent: Thursday, October 12, 2006 7:11 AM
To: Johncie Wingard
Subject: RE: USADA Request

Dear Mr. Wingard,

As a follow-up to your e-mail hereunder, we inform you that our answer will be provided during the next week.

We appreciate your kind collaboration and thank you for your patience.

Best Regards

Delphine Lautenschlager
Assistante
Service Antidopage
Antidoping Services
Union Cycliste Internationale
CH - 1860 Aigle

Tél : +41-24.468.58.11
Fax : +41-24.468.58.12
www.uci.ch

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

From: Johncie Wingard [mailto:jwingard@usantidoping.org]
Sent: jeudi, 5. octobre 2006 19:26
To: Lautenschlager Delphine - UCI
Subject: USADA Request

Ms. Lautenschlager – I am following up on the emails that I sent to you on Sept. 12, 2006. I have included the language from those emails below for your convenience. Could you please update me on the status of our requests? We greatly appreciate your assistance in this matter. Thank you.

Ms. Lautenschlager – Travis asked me to follow up with you after he talked to you this morning. Christian Varin provided us with some documents for this rider in correspondence dated 9 August, 2006. Those documents included a chart of all of the samples collected by UCI for this rider including sample number, date, event and T/E ratio if available. We received the Doping Control Official Record and lab test results for the following samples:

874535
876013
874285
872364
872357
876004
995462
994203

994277
994276
994075
994080
994171

We would appreciate receiving the Doping Control Official Record and laboratory test results for all of the other samples listed in the chart which would be for all the samples collected from 1999 through 2004. If you could please provide those documents to us, we will contact the laboratories directly for the steroid profile data.

We greatly appreciate your assistance in this matter. If you have any questions, please do not hesitate to contact Travis directly.

Ms. Lautenschlager – after closer review of the documents sent by Mr. Varin, besides all of the samples from 1999-2004, we also still need Doping Control Official Records and laboratory reports for several samples in 2005 & 2006 as listed below:

920460
920462
951826
951875
951828
951866
951872
951792
951789
951787
951790

I apologize for omitting these from my earlier message.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

Johncie Wingard

From: Lautenschlager Delphine - UCI [Delphine.Lautenschlager@uci.ch]
Sent: Wednesday, October 18, 2006 7:16 AM
To: Johncie Wingard
Subject: RE: USADA Request
Attachments: authorization.pdf; Algarve.pdf; Amgen Tour.pdf; Georgia.pdf; Paris-nice.pdf; Vuelta.pdf

Dear Mr. Wingard and Mr. Tygart,

Please, find herewith attached a copy of the anti-doping forms and a copy of the laboratory's results concerning the file 29/06. (2004-2005-2006)

Moreover, you will find an authorization per laboratory authorizing you to request the steroid profiles.

In my phone call with Mr. Tygart, I explained that it is kind of difficult for us to find the forms before 2004. If this information is really necessary we will have to look out in our old files and of course this will take more time.

Best regards,

Delphine Lautenschlager
Assistante
Service Antidopage
Antidoping Services
Union Cycliste Internationale
CH - 1860 Aigle

Tél : +41-24.468.58.11
Fax : +41-24.468.58.12
www.uci.ch

IMPORTANT NOTICE :

This message contains confidential information and is intended only for the individual named herein. If you are not the herein named addressee you should not disseminate, distribute copy or otherwise make use of this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake, and delete this e-mail from your system.

From: Johncie Wingard [mailto:jwingard@usantidoping.org]
Sent: jeudi, 5. octobre 2006 19:26
To: Lautenschlager Delphine - UCI
Subject: USADA Request

Ms. Lautenschlager – I am following up on the emails that I sent to you on Sept. 12, 2006. I have included the language from those emails below for your convenience. Could you please update me on the status of our requests? We greatly appreciate your assistance in this matter. Thank you.

Ms. Lautenschlager – Travis asked me to follow up with you after he talked to you this morning. Christian Varin provided us with some documents for this rider in correspondence dated 9 August, 2006. Those documents included a chart of all of the samples collected by UCI for this rider including sample number, date, event and T/E ratio if available. We received the Doping Control Official Record and lab test results for the following samples:

874535
876013
874285
872364

872357
876004
995462
994203
994277
994276
994075
994080
994171

We would appreciate receiving the Doping Control Official Record and laboratory test results for all of the other samples listed in the chart which would be for all the samples collected from 1999 through 2004. If you could please provide those documents to us, we will contact the laboratories directly for the steroid profile data.

We greatly appreciate your assistance in this matter. If you have any questions, please do not hesitate to contact Travis directly.

Ms. Lautenschlager – after closer review of the documents sent by Mr. Varin, besides all of the samples from 1999-2004, we also still need Doping Control Official Records and laboratory reports for several samples in 2005 & 2006 as listed below:

920460
920462
951826
951875
951828
951866
951872
951792
951789
951787
951790

I apologize for omitting these from my earlier message.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

UNION CYCLISTE INTERNATIONALE
CH 1860 Aigle / Suisse
✆ : +41 24 468 58 11 – Fax : +41 24 468 58 12 – e-mail : anne.gripper@uci.ch

AUTHORIZATION

The UCI Anti-Doping Commission authorizes the U.S. Anti-Doping Agency (USADA) to request to Agence française de lutte contre le dopage, Département des Analyses, Châtenay-Malabry, France the steroid profiles for the following sample:

289130

Anti-Doping Services,

Anne Gripper

Anne Gripper, Manager

UNION CYCLISTE INTERNATIONALE
CH 1860 Aigle / Suisse
✆ : +41 24 468 58 11 – Fax : +41 24 468 58 12 – e-mail : anne.gripper@uci.ch

AUTHORIZATION

The UCI Anti-Doping Commission authorizes the U.S. Anti-Doping Agency (USADA) to request to UCLA Olympic Analytical Laboratory, Los Angeles, USA the steroid profiles for the following samples:

920460
920462
951826
951875
951828
951866
951872
951792
951789
951787
951790

Anti-Doping Services,

Anne Gripper

Anne Gripper, Manager

UNION CYCLISTE INTERNATIONALE
CH 1860 Aigle / Suisse
✆ : +41 24 468 58 11 – Fax : +41 24 468 58 12 – e-mail : anne.gripper@uci.ch

AUTHORIZATION

The UCI Anti-Doping Commission authorizes the U.S. Anti-Doping Agency (USADA) to request to Laboratorio de Control del Dopaje del Consejo Superior de Deportes Madrid, Spain the steroid profiles for the following samples:

342599
347883
342856
342850
348369

Anti-Doping Services,

Anne Gripper

Anne Gripper, Manager

UNION CYCLISTE INTERNATIONALE
CH 1860 Aigle / Suisse
✆ : +41 24 468 58 11 – Fax : +41 24 468 58 12 – e-mail : anne.gripper@uci.ch

AUTHORIZATION

The UCI Anti-Doping Commission authorizes the U.S. Anti-Doping Agency (USADA) to request to Laboratório e Análises e Dopagem Instituto do Desporto de Portugal, Lisbonne, Portugal the steroid profiles for the following sample:

277821

Anti-Doping Services,

Anne Gripper

Anne Gripper, Manager

Johncie Wingard

m: Johncie Wingard
Sent: Tuesday, November 21, 2006 10:22 AM
To: 'areyes@ucla.edu'
Attachments: Ltr FROM UCLA to USADA 2.doc

Ana – I am following up on a previous request for steroid profile data on two samples (1501850 and 497104). They were follow-up samples on the same athlete for whom steroid data was previously provided. Attached is a sample letter we would like you to include with the data. We would appreciate receiving the data as soon as possible. Thanks very much for your assistance and if you have any questions, please contact Travis directly.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

EXAMPLE

[Date]

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: Sample #s: 1501850 and 497104

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples are accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Don Catlin
Director

Enclosure

Johncie Wingard

From: Ana Reyes [afreyes@ucla.edu]
Sent: Thursday, November 30, 2006 1:52 PM
To: Johncie Wingard
Cc: 'Don Catlin'
Subject: RE: Scanned image from MX-4501N Color

Dear Johncie,

Thank you very much for sending this information. It appears that the e-mail was sent around the time Paul Scott left the laboratory. He did not pass on this request to me or anyone in the laboratory.

My sincere apologies that this request was not addressed.

Best regards,

Ana

Ana Reyes
Service/Quality Manager
UCLA Olympic Analytical Laboratory
Main Phone: (310) 825-2635
FAX: (310) 206-9077
E-mail:afreyes@ucla.edu

-----Original Message-----
From: Johncie Wingard [mailto:jwingard@usantidoping.org]
Sent: Thursday, November 30, 2006 9:34 AM
To: Ana Reyes
Subject: FW: Scanned image from MX-4501N Color

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

UCLA OLYMPIC ANALYTICAL LABORATORY

Don H. Catlin, M.D., Director
2122 Granville Avenue
Los Angeles, CA 90025-6106
Tel: (310) 825-2635
Fax: (310) 206-9077

DATE: November 30, 2006

TO: TRAVIS TYGART / JOHNCIE NINHAM
GENERAL COUNSEL
USADA

FAX#: (719) 785-2028

FROM: ANA REYES
SERVICE/QUALITY MANAGER

NUMBER OF PAGES (including cover page): 3

MESSAGE:

This message is intended only for the party to which it is addressed. It contains confidential information. If you have received this message in error, please contact the sender at one of the above numbers immediately. Thank you.

UNIVERSITY OF CALIFORNIA, LOS ANGELES

UCLA

BERKELEY • DAVIS • IRVINE • LOS ANGELES • MERCED • RIVERSIDE • SAN DIEGO • SAN FRANCISCO

SANTA BARBARA • SANTA CRUZ

UCLA OLYMPIC ANALYTICAL LABORATORY
DEPARTMENT OF PHARMACOLOGY
UCLA SCHOOL OF MEDICINE
2122 GRANVILLE AVENUE
LOS ANGELES, CALIFORNIA 90025

PHONE (310) 825-2635
FAX (310) 206-9077

November 30, 2006

Travis T. Tygart
General Counsel
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: Sample #s: 1501850 and 497104

Dear Mr. Tygart:

Pursuant to your request, attached please find steroid profile data on the above referenced samples. Additionally, I have reviewed the files related to these samples and hereby confirm the following:

1. These samples were accompanied by appropriate transport chain of custody documentation when they were received at our laboratory.

2. These samples were properly sealed when they were received at our laboratory.

3. Our laboratory maintained an appropriate chain of custody for the samples while they were in the custody of our laboratory.

4. Our laboratory followed its regular WADA approved process in analyzing these samples.

5. The screen results for these samples are accurate.

If you have any questions or concerns about this matter, please contact me directly.

Sincerely,

Ana Reyes
Service/Quality Manager
UCLA Olympic Analytical Laboratory

cc: Dr. Catlin

UNIVERSITY OF CALIFORNIA, LOS ANGELES

UCLA

BERKELEY · DAVIS · IRVINE · LOS ANGELES · MERCED · RIVERSIDE · SAN DIEGO · SAN FRANCISCO

SANTA BARBARA · SANTA CRUZ

UCLA OLYMPIC ANALYTICAL LABORATORY
DEPARTMENT OF PHARMACOLOGY
UCLA SCHOOL OF MEDICINE
2122 GRANVILLE AVENUE
LOS ANGELES, CALIFORNIA 90025

PHONE (310) 825-2635
FAX (310) 206-9077

CONFIDENTIAL

VIA TELEFAX

November 30, 2006

Travis Tygart
General Counsel
United States Anti-Doping Agency
FAX # (719) 785-2028

Re: Request for steroid profile on USADA
Sample # 1501850 = UCLA 94T03 and
Sample # 497104 = UCLA 9S604

Dear Mr. Tygart,

Below are the data that you requested. Please keep in mind that the data are from our steroid screen assay which is optimized for quantitating testosterone. The screen is not optimized for epitestosterone (E) but it will provide reasonable approximations of the E concentration. The assay will not provide reliable estimates of the concentrations of androsterone, etiocholanolone, or the 11-betas.

Sample #	UCLA Code	pH	S.G.	Testosterone Conc.(ng/mL)	Epitestosterone Conc. (ng/mL)	Androsterone Conc.(ng/mL)	Etiocholanolone Conc. (ng/mL)	11b-OHA Conc. (ng/mL)	11b-OHE Conc. (ng/mL)	T/E Ratio
1501850	94T03	7.0	1.006	8	5	532	1284	186	46	1.5
497104	9S604	6.0	1.023	73	83	5444	10163	1291	703	0.9

Please contact me should you have any questions.

Sincerely,

Ana Reyes
Service/Quality Manager
UCLA Olympic Analytical Laboratory

cc: Dr. Catlin

Johncie Wingard

m: Ana Reyes [afreyes@ucla.edu]
Sent: Wednesday, December 06, 2006 5:44 PM
To: Johncie Wingard
Cc: 'Don Catlin'; 'Michael Sekera'
Subject: RE: Steroid Profile Data

Hi Johncie,

Here is the additional information you requested. I have added a disclaimer with the data.

	5αAdiol (ng/mL)	5βAdiol (ng/mL)
Sample # 1501850= UCLA94T03	7	88
Sample # 497104=UCLA 9S604	271	870

DISCLAIMER: the diol data are derived from a screening assay that does not include a quantitative standard for either diol. No attempt is made to repeat the injection or analysis if the diol concentrations seem high or low. The quantitation is based on the deuterated testosterone which is added to the sample. It is assumed that the GC/MS response of the diols and T are the same as T.

Ana

Ana Reyes
Se e/Quality Manager
J Olympic Analytical Laboratory
Main Phone: (310) 825-2635
FAX: (310) 206-9077
E-mail:afreyes@ucla.edu

From: Johncie Wingard [mailto:jwingard@usantidoping.org]
Sent: Wednesday, December 06, 2006 1:26 PM
To: Ana Reyes
Subject: RE: Steroid Profile Data

Ana – attached is the letter and chart that was sent to us a few months back and it contains those amounts. I know that you do not typically provide those amounts but, if I recall correctly, they are always included when we get further profile data from your lab.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

From: Ana Reyes [mailto:afreyes@ucla.edu]

Sent: Wednesday, December 06, 2006 2:06 PM
To: Johncie Wingard
Subject: RE: Steroid Profile Data

Hi Johncie,

I received this e-mail. Perhaps your earlier e-mail did not go through because we had intermittent problems with e-mail the past week.
Sorry but we do not provide 5αAdiol and the 5βAdiol amounts in the steroid profile info that we send to USADA.

Thanks,
Ana

Ana Reyes
Service/Quality Manager
UCLA Olympic Analytical Laboratory
Main Phone: (310) 825-2635
FAX: (310) 206-9077
E-mail:afreyes@ucla.edu

From: Johncie Wingard [mailto:jwingard@usantidoping.org]
Sent: Wednesday, December 06, 2006 12:59 PM
To: Ana Reyes
Subject: FW: Steroid Profile Data

Ana - If this is a duplicate email I apologize, but I am not sure the one I just sent went thorough. Can you please send the 5αAdiol and the 5βAdiol amounts for the two samples that you sent steroid profile data on last week? Please let me know that you received this message. Thank you.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

From: Johncie Wingard
Sent: Friday, December 01, 2006 8:37 AM
To: 'Ana Reyes'
Subject: Steroid Profile Data

Ana - thank you for the fax yesterday regarding our request for steroid profile data. Is it possible to also get the 5αAdiol and the 5βAdiol amounts for each sample??

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

UNION CYCLISTE INTERNATIONALE

Président

Agence Française de Lutte contre le Dopage (AFLD)
39, rue Saint Dominique
75007 PARIS
FRANCE

Uniquement par fax: 0033.1.40.62.77.39

Aigle, le 18 Janvier 2007
Ref: President / al

Floyd Landis

Monsieur le Président,

Nous avons appris que l'AFLD avait convoqué M. Floyd Landis dans le cadre d'une procédure disciplinaire qui a été ouverte à son encontre suite à un résultat analytique anormal lors du Tour de France 2006.

Nous avons compris qu'il s'agit de la procédure disciplinaire suivant la Loi française en vigueur au moment des faits (athlète non licencié auprès d'une fédération nationale française).

Quand cette Loi a été introduite, des entretiens ont eu lieu entre le CPLD et l'UCI et une pratique s'est installée de commun accord suivant laquelle le CPLD attendrait le résultat de la procédure internationale suivant les règles de l'UCI avant de prendre sa propre décision, suivant une procédure simplifiée évitant à l'athlète les inconvénients d'une double procédure, si le CPLD pouvait se rallier à la décision prise par la fédération nationale du coureur.

Or, le 26 juillet 2006, l'UCI a ouvert une procédure disciplinaire contre M. Landis. Comme le prévoit le règlement antidopage de l'UCI, le dossier est traité par l'Agence Antidopage des Etats-Unis (USADA) pour le compte de la fédération nationale de cyclisme du coureur.

Il en résulte que la même affaire est traitée par deux instances différentes et que l'athlète se voit contraint de se défendre devant chacune d'elles, en l'occurrence dans des continents différents, dans des langues différentes et selon des procédures différentes.

C'est une situation qu'a voulu exclure justement le Code Mondial Antidopage, auquel la France a adhéré en signant la Déclaration de Copenhague et en adoptant la Loi du 5 avril 2006.

Le Code Mondial Antidopage donne la compétence pour des cas comme celui de M. Landis à la fédération internationale concernée. Cette règle se retrouve également dans la Loi française du 5 avril 2006.

Nous savons bien que l'AFLD est compétente suivant les dispositions transitoires de la Loi du 5 avril 2006. Néanmoins, pour les motifs invoqués ci-dessus, nous avons l'honneur de vous demander que la procédure devant l'AFLD soit remise jusqu'à ce que la procédure prévue par le règlement de l'UCI soit terminée, même si, nous le reconnaissons, cette procédure prend beaucoup de temps.

Cette demande ne nous paraît pas contraire à la Loi française mais s'inscrit, par contre, dans le but de la Loi comme dans celui du Code Mondial Antidopage.

En vous remerciant par avance de l'attention que vous voudrez bien porter à la présente, je vous prie de croire, Monsieur le Président, à l'expression de mes salutations distinguées.

P W McQ

Pat McQuaid
President

Cc: AMA, M. David Howman (fax. + 1.514.904.8771)
USADA, M. Travis Tygart (fax: +1.719.785.2001)
M. Howard L. Jacobs, conseil de M. Floyd Landis (fax : +1.818.292.8736)

UNITED STATES ANTI-DOPING AGENCY
Doping Control Official Record

FLOYD LANDIS — 12-08-02 — 6 40 AM (PM)

ETE / OR PLACE / OR EVENT — NOTIFICATION DATE — TIME

Pursuant to USADA Regulations:

- You are required to be drug tested. Signature on this form constitutes your consent to such testing.
- You have a maximum of 60 minutes from time of notification to arrive with your Chaperone at the doping control station/area, unless being tested under the Out-of-Competition conditions.
- **Refusal to cooperate or failure to report within the given time limit, may subject you to sanctions consistent with a positive drug test.**
- Consumption of any fluid or food even if provided by the Chaperone or a Doping Control Officer is at your own risk.
- You may have a representative accompany you through the testing process, but such representative may not witness the voiding.

Steve Voskuhl — Floyd Landis — Floyd Landis

Notifying Chaperone's Name — Athlete Signature — (Print Name)

ATHLETE INFORMATION

FLOYD LANDIS — 12-08-02 — 6 40 AM (PM)

Athlete Name (PRINT) — ARRIVAL DATE — TIME

40371 Miklich Dr. — 10/14/1975

Street Address — Date of Birth (month / day / year)

Murrieta CA 92563 — California D2203288

City, State, Zip Code, COUNTRY — I.D. # (or "n/a" if Event)

909-461-8462

Phone

LABORATORY/SPECIMEN INFORMATION

☐ Event ☒ Out-of-Competition ☐ Other _____

☐ Full Menu Test ☒ Partial Menu Test

12-08-02 — 6 53 AM (PM)

SAMPLE COLLECTION DATE — TIME

OOC

Site I.D. # ("OOC" if Out-of-Competition)

4 6 6 1 9 3 — ☒ MALE ☐ FEMALE

SAMPLE CODE NUMBER

SPECIFIC GRAVITY and pH levels within range ☒ YES ☐ NO

Specific Gravity: ≥1.010 (≥1.005 if measured by refractometer)
pH: Not less than five and not greater than seven (5≥ x ≤7)

Cycling

Sport

Austin TX

Competition Name (or City/State if OOC)

SUBSEQUENT SPECIMEN

_____ AM PM

SUBSEQUENT SAMPLE CODE NUMBER — TIME

SPECIFIC GRAVITY and pH levels within range ☐ YES ☐ NO

Declaration of medications and nutritional supplements taken during the preceding three (3) days (write "none" if none declared):

Medication / Supplement	Dose	Last Taken	Medication / Supplement	Dose	Last Taken
NONE					

SIGNATURES

By signing below I certify that: (i) I selected a sealed collection beaker and a sample kit from the supply provided, opened and visually checked that they were empty and clean, sealed them securely and confirmed that the numbers on the sample kit and bottles were identical to the numbers written on this Doping Control Official Record; (ii) the Doping Control Officer did not handle my sample except when permitted by me; (iii) no irregularities occurred in the sample collection process; and (iv) this information is not being provided to USADA pursuant to a doctor/patient relationship and is not to be considered a confidential medical record.

Athlete Signature — Floyd Landis (Print Name) — N/A Language Specialist Signature (or "n/a" if none) — (Print Name)

The following individuals were present during the sample collection and/or processing procedure(s).

Jerry L. Mom, Jr — (Print Name) — N/A Subsequent Chaperone (or "n/a" if none) — (Print Name)

Chaperone Signature

Doping Control Officer Signature — Steve Voskuhl (Print Name) — N/A Athlete Representative (or "n/a" if none)

☐ Partial Sample Form ☐ Supplementary Report Form

USADA 0612

IOC Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave Los Angeles CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL
DRUG TESTING REPORT USADA5079

Terry Madden
The United States Anti-Doping Agency
2550 Tenderfoot Hill St., Suite 200
Colorado Springs, CO 80906

UCLA code: YWI
Date of laboratory receipt: 12/10/02

Number of samples: 16
Site ID: OOC

The laboratory analyzed the specimens listed below for the IOC out-of-competition list of prohibited substances: using methods 1001 anabolic and masking agents by GC-MS, 2001 diuretics by GC-MS, and 6001 peptide hormones (hCG) by immunoassay (males only).

The custody (C) is intact if indicated by 'Y'.

UCLA Code	Specimen#	Results	C
YWI08	466193	NEGATIVE	Y

Sanja Starcevic, Ph D — S. Starcevic — 12/16/02

Certifying Scientist — Signature — Date

12/17/02

This report shall not be reproduced, except in full, without the written approval of the laboratory.

IOC Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave Los Angeles CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL
DRUG TESTING REPORT USADA5079

Site ID: OOC

UCLA Code	Specimen #	Site pH	Lab pH	Site SG	Lab SG
YWI08	466193	N	7.0	N	1.020

This report shall not be reproduced, except in full, without the written approval of the laboratory.

UNITED STATES ANTI-DOPING AGENCY
Doping Control Official Record

Floyd LANDIS — ...LETE / OR PLACE / OR EVENT

11-26-03 — NOTIFICATION DATE

430 AM (PM) — TIME

Pursuant to USADA Regulations:

- You are required to be drug tested. Signature on this form constitutes your consent to such testing.
- You have a maximum of 60 minutes from time of notification to arrive with your Chaperone at the doping control station/area, unless being tested under the Out-of-Competition conditions.
- You should bring a photo ID with you to the Doping Control station/area.
- **Refusal to cooperate or failure to report within the given time limit, may subject you to sanctions consistent with a positive drug test.**
- Consumption of any fluid or food even if provided by the Chaperone or a Doping Control Officer is at your own risk.
- You may have a representative accompany you through the testing process, but such representative may not witness the voiding.

Thomas R McVay — Notifying Chaperone's Name (Print)

[signature] — Athlete Signature

Floyd Landis — (Print Name)

ATHLETE INFORMATION

Floyd LANDIS — Athlete Name (PRINT)

11-26-03 — ARRIVAL DATE

430 AM (PM) — TIME

23356 Bishop Rd — Mailing Address

10-14-75 — Date of Birth (month / day / year)

MURRIETA CA 92562 USA — City, State, Zip Code, COUNTRY

CA — Photo Identification

DL — Type

D2203288 — No.

(909) 461-8462 — Phone

LABORATORY/SPECIMEN INFORMATION

☐ Event ☒ Out-of-Competition ☐ Other _____

☐ Full Menu Test ☒ Partial Menu Test

11-26-03 — SAMPLE PROCESSING DATE

455 AM (PM) — TIME

OOC — Site I.D. # ("OOC" if Out-of-Competition)

4	2	6	3	1	5

SAMPLE CODE NUMBER

☒ MALE ☐ FEMALE

Cycling — Sport

SPECIFIC GRAVITY and pH levels within range ☒ YES ☐ NO

Specific Gravity: ≥1.010 (≥1.005 if measured by refractometer)
pH: Not less than five and not greater than seven (5≥ x ≤7)

MARRIETTA CA — Event = Competition Name OOC = City/State

SUBSEQUENT SPECIMEN

SUBSEQUENT SAMPLE CODE NUMBER

AM PM _____ TIME

SPECIFIC GRAVITY and pH levels within range ☐ YES ☐ NO

Declaration of medications and nutritional supplements taken during the preceding three (3) days (write "none" if none declared):

Medication / Supplement	Dose	Date Last Taken	Medication / Supplement	Dose	Date Last Taken
RhinoCORT	100µg	11/25/03			
Serevent	1 dose	?			
Injection for Tendon (L)		9/18/03			

SIGNATURES

By signing below I certify that: (i) I selected a sealed collection container and a sample kit from the supply provided, opened and visually checked that they were empty and clean, sealed them securely and confirmed that the numbers on the sample kit and bottles were identical to the numbers written on this Doping Control Official Record; (ii) the Doping Control Officer did not handle my sample except when permitted by me; (iii) no irregularities occurred in the sample collection process; and (iv) this information is not being provided to USADA pursuant to a doctor/patient relationship and is not to be considered a confidential medical record.

[signature] — Athlete Signature

Floyd Landis — (Print Name)

N/A — Language Specialist Signature (or "n/a" if none)

(Print Name)

The following individuals were present during the sample collection and/or processing procedure(s).

[signature] — Chaperone Signature

Thomas R McVay — (Print Name)

N/A — Subsequent Chaperone (or "n/a" if none)

(Print Name)

[signature] — Doping Control Officer Signature

Thomas R McVay — (Print Name)

N/A — Athlete Representative (or "n/a" if none)

USADA 0615

IOC Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave Los Angeles CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL
DRUG TESTING REPORT USADA5812

Terry Madden
The United States Anti-Doping Agency
2550 Tenderfoot Hill St., Suite 200
Colorado Springs, CO 80906

UCLA code: 1ER
Date of laboratory receipt: 12/01/03
Number of samples: 9
Site ID: OOC

The laboratory analyzed the specimens listed below for the IOC out-of-competition list of prohibited substances: using methods 1001 anabolic and masking agents by GC-MS, 2001 diuretics by GC-MS, and 6001 peptide hormones (hCG) by immunoassay (males only). The specimens were also analyzed for tetrahydrogestrinone.

The custody (C) is intact if indicated by 'Y'.

UCLA Code	Specimen#	Results	C
1ER03	476315	NEGATIVE	Y

JAN 0 8 2004

Yulia Kucherova — 1-8-04

Certifying Scientist — Signature — Date

This report shall not be reproduced, except in full, without the written approval of the laboratory.

IOC Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave Los Angeles CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL
DRUG TESTING REPORT USADA5812

Site ID: OOC

UCLA Code	Specimen #	Site pH	Lab pH	Site SG	Lab SG
1ER03	476315	N	5.0	N	1.023

This report shall not be reproduced, except in full, without the written approval of the laboratory.

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☒
 Hors compétition / Out of competition: ☐
2. Sexe: Masculin / Male ☒
 Sex: Féminin / Female ☐
3. Date: 22/2/2004
4. Lieu / Place: MALHÃO
5. Course (nom, étape): VOLTA AO ALGARVE - 5ª ET.
 Race (name, stage):
6. Discipline: ROUTE
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD
 First name:

 Adresse du coureur: 23356 BISHOP ROAD, MURRIETA, CA 92562, USA
 Address of the rider:
8. Code UCI: USA 19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA CYCLING
 National federation which delivered the license:
10. Numéro de licence: B0020272
 License number:
11. Tiré au sort: Oui / Yes ☐
 Random: Non / No ☒
12. Heure de présentation: 15,50
 Presentation time:
13. Heure de prélèvement: 15,52
 Time of sampling:
14. Code des flacons: 277821 A+B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris: —
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé: SERRAVENT
Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☐
 Therapeutic use exemption (TUE) Non / No ☒
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

—

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur: JOHAN DEMECHELEER
 Assistant: (nom/name) (signature)
20. Médecin contrôleur: DR. VÍTOR PALMEIRA
 Examining doctor: (nom/name) (signature)
21. Inspecteur antidopage: JOSÉ SOARES
 Antidoping inspector: (nom/name) — (signature)

USADA 0618

Instituto do Desporto de Portugal

Laboratório de Análises e Dopagem

Av. Prof. Egas Moniz Desportiva (Estádio Universitário)
1600-190 Lisbon, PORTUGAL
Tel: +351 217969073 Fax: +351 217977529

TEST REPORT

CONTROL IDENTIFICATION

Client	**CONSELHO NACIONAL ANTIDOPAGEM**						
Federation	INTERNATIONAL CYCLING UNION		**Sport**	CYCLING			
Address	ANTIDOPING SERVICES CH - 1860 AIGLE SWITZERLAND						
Event	30.ª VOLTA AO ALGARVE	**Date of collection**	22 / 02 / 04	**Date of receipt**	25 / 02 / 04	**Code**	GENIAL

SAMPLE IDENTIFICATION

Type of samples	**Urine** ☒	**Blood** ☐	**Sample "A"** ☒	**Sample "B" (B analysis)** ☐

Laboratory number	**Sample number**	**In competition** ☒	**Out-of-competition** ☐
0225	277821		

The containers, bottles and samples were received in good conditions and did not show any sign of manipulation which could possible affect the validity of the results. The Laboratory is not responsible for the sample collection.

TEST RESULTS

Results:

Period of performance of the tests: 25 / 02 / 04 to 22 / 03 / 04

GATIVE SAMPLES	**277821** ✓			

For the samples mentioned above, no forbidden or restricted substances were detected, neither evidences of the use of forbidden doping methods specified in regulations relevant to the sport in question.

The screening procedures performed were the following:

PTEC-LADB-100	PTEC-LADB-101	PTEC-LADB-103	PTEC-LADB-104	PTEC-LADB-106	PTEC-LADB-107h*	PTEC-LADB-107a	PTEC-LADB-107g*	PTEC-LADB-108	PTEC-LADB-109*

Observations:

The sample "A" 277824 is suspected to contain a diuretic. The result of the confirmation is not available at this moment.

Scientific Director

Lisbon, 23/03/2004

Dr. Douwe de Boer

This test report shall not be reproduced except in full, without written approval of the laboratory.
These results relate only to the items tested.

CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS Floyd A.

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 Bishop Rd MURRIETA CA 92562

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : B 002/0272/

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14.10.75

Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : CYCLISME SUR ROUTE

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 3ème étape ROANNE

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 09/07/2004

Heure de remise de la notification : 16h50

Lieu du contrôle / Place of test : MOBILE HOME ORGANISATION ROANNE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested LANDIS

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : BOUSQUET L.

Nom et prénom du médecin / Name of doctor : (en toutes lettres) KHENNOUF Abbas

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 16h50

Date du prélèvement / Sampling date : 09/07/2004

Heure du prélèvement / Sampling time : 16h55

Identification / Identification :
- Licence / Licence : ☐
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 289130

Quantité totale d'urine, ml / Total amount of urine, ml : 125 mL

Densité / Specific Gravity : 1,025

pH / pH : 6

Échantillon insuffisant / Insufficient sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal : ☐ Oui ☐ Non

Empêchement / Obstacle : ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)
SERESTA* Rp 26/04/2004
Docteur EQUIPE US POSTAL

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : RAS

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : Signature of person tested :

Signature du Médecin : Signature of Doctor :

Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

3010661 1:0.8 CFB
NATIONAL

LABORATOIRE NATIONAL
DE DEPISTAGE DU DOPAGE

cofrac
ESSAIS
ACCRÉDITATION
N° 1-1174
PORTÉE
COMMUNIQUÉE
SUR DEMANDE

Châtenay-Malabry, le 23 mars 2004

21941

RAPPORT D'ANALYSE N° 61/03

Votre Demande

Prélèvements du Dr KHENNOUF

Organisme : **Union Cycliste Internationale**
Épreuve et lieu : **Paris-Nice 3ème étape contrôle effectué à Roanne**
Date : **09/03/2004**

Réception des échantillons d'urine :

Date : **11/03/2004**
Nombre d'échantillons : **4 (/ 4 échantillons reçus)**
Nombre de rapports d'analyse : **1**
Référence des échantillons : **289130**

Résultats (Cf référentiels en vigueur : AMA / réglementation nationale : Contrôle en compétition)

Analyses terminées le : **23/03/2004**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES01, ES02, ES04, ES05*) et liquide (ES03, ES03 B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : **Recherche de substances dopantes négative**

Pour le Directeur
Françoise LASNE
Responsable Technique

Destinataires :

- Union Cycliste Internationale – C. VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique– 75700 PARIS)

* Hors portée d'accréditation.

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

USADA 0621

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE

LABORATOIRE NATIONAL
DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°61/03

Résultats des rapports T/E

Référence des échantillons	T/E
289130	0.9

Pour le Directeur
Françoise LASNE
Responsable Technique

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☒
 Hors compétition / Out of competition: ☐
2. Sexe: Masculin / Male ☒
 Sex: Féminin / Female ☐
3. Date: 6.9.2004
4. Lieu / Place: LEON (ESP)
5. Course (nom, étape): 1ère étape (C.L.M. par équipes)
 Race (name, stage): VUELTA A ESPAÑA
6. Discipline: ROUTE
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD
 First name:
 Adresse du coureur: 23356 BISHOP RD MURRIETA CA 92562 (USA)
 Address of the rider:
8. Code UCI: USA 19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: U.S.A.
 National federation which delivered the license:
10. Numéro de licence: 10020278
 License number:
11. Tiré au sort: Oui / Yes ☒
 Random: Non / No ☐
12. Heure de présentation: 17 h 59
 Presentation time:
13. Heure de prélèvement: 18 h 10'
 Time of sampling:
14. Code des flacons: 342599 A+B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris:
 Pharmaceutical drugs taken by rider: IN ST ANTONIUS HOSPITAL ON 07/08/2004 DIPIRIDAMOR AND NOVALGINE DOCUMENTS ATTACHED

Contenu du livret de santé:
Contents of the health booklet: 26/01/04 SERETIDE (ASMA BRONQUIAL)
08/04/2004 POSITON CREMA (TRIAMCINOLONA AC.) DERMATITIS PIES Y MANOS
22/04/04 DIAROPHA (BETAMETASONA) TENDINITIS RODILLA DERECHA

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☒
 Therapeutic use exemption (TUE) Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur: DIEGO VAN ESCANDE
 Assistant: (nom/name) (signature)
20. Médecin contrôleur: FERNANDO GANCHEGUI
 Examining doctor: (nom/name) (signature)
21. Inspecteur antidopage: VANDEVYVERE MARC (BEL) COMMIS. INT. U.C.I.
 Antidoping inspector: INSP. ANTIDOPAGE (nom/name) (signature)

USADA 0623

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL COMITÉ OLÍMPICO INTERNACIONAL

PROCÈS-VERBAL: 041185 DONNÉES GÉNÉRALES

CONTRÔLE: EN COMPETICIÓN	**Organisme expéditeur:** UCI, CH 1860 Aigle, SUISSE 0
Sport: CICLISMO	**Modalité Sportive:** RUTA
Journée, étape ou match: 1ª ETAPA	**Lieu du contrôle:** LEON
Date de la prise d'échantillons: 04/09/2004	**Date de réception:** 06/09/2004
Compétition, épreuve ou activité sportive: VTA. CICLISTA A ESPAÑA	**Date début d'analyse:** 06/09/2004
	Date fin d'analyse: 07/09/2004

Remarques:

PROCÈS-VERBAL: 041185 DONNÉES DES ÉCHANTILLONS "A"

Registre du Laboratoire	Code du cachet	Code du flacon "A"
04126898A	CSD 342599	CSD A342599

PROCÈS-VERBAL: 041185 RÉSULTATS DES ANALYSES

Les échantillons d'urine signalés ci-dessus ont été analysés dans ce Laboratoire conformément aux procédés: PNT-DE-01, PNT-DE-02, PNT-DE-03, quantification de hCG* et détection du glucocorticostéroïdes* (PNT-DE-07), sans qu'aucune substance pouvant être à l'origine d'un résultat positif de contrôle du dopage n'y ait été détectée, que ce soit qualitativement ou quantitativement.

* L'essai de quantification de hCG et détection du glucocorticostéroïdes ne sont pas compris dans le domaine d'application de l'accreditation du laboratoire.

Il est déclaré que les résultats obtenus ne concernent que les échantillons ayant été soumis à l'analyse.

Madrid le 08-sep-04

Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES LABORATORIO CONTROL DOPAJE MADRID

Dr. Agustín Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinf.rodriguez@csd.mec.es

USADA 0624

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

ENAC
Nº 230/LE606

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL C.I.O / W.A.D.A.

PROCÈS-VERBAL: 041185 **RAPPORT T/E**

Enregistrement du laboratoire	Code du flacon "A"	T/E	Procédé
04126898A	CSD A342599	1,7	PNT-DE-03

Il est déclaré que les résultats obtenus ne concernent que les échantillons ayant été soumis à l'analyse.

Madrid, 8 de sep-04
Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES
LABORATORIO CONTROL DOPAJE
MADRID

Dr. Agustín Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinf.rodriguez@csd.mec.es

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☒
 Hors compétition / Out of competition: ☐
2. Sexe / Sex: Masculin / Male ☒ Féminin / Female ☐
3. Date: 11.9.2004
4. Lieu / Place: ALMUÑÉCAR (ESP)
5. Course (nom, étape): 5ème étape: C.L.M.
 Race (name, stage): VUELTA A ESPAÑA
6. Discipline / Discipline: ROUTE
7. Nom du coureur / Surname of rider: LANDIS
 Prénom / First name: FLOYD
 Adresse du coureur / Address of the rider: BISHOP ROAD 23356 CA 92562 MURRIETA (USA)
8. Code UCI / UCI Code: USA 19750714
9. Fédération nationale ayant délivré la licence / National federation which delivered the license: U.S.A
10. Numéro de licence / License number: 13002272
11. Tiré au sort / Random: Oui / Yes ☐ Non / No ☒
12. Heure de présentation / Presentation time: 17h40'
13. Heure de prélèvement / Time of sampling: 18h05'
14. Code des flacons / Bottle codes: 342856 A+B
15. Heure de refus du coureur / Time of rider's refusal:
16. Médicaments pris / Pharmaceutical drugs taken by rider:

Contenu du livret de santé / Contents of the health booklet:
26/01/04 Sérevent (Asma bronquial)
8/04/04 Triamcinolona Act (Dermatitis en pie y muñeca)
22/04/04 Betametasona (Tendinitis rotula dcha)
9/9/04 Triamcinolona (Tendinitis)

17. Autorisation d'usage à des fins thérapeutiques (AUT) / Therapeutic use exemption (TUE): Oui / Yes ☒ Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement. / Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie / Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur / Assistant: (nom/name) (signature)
20. Médecin contrôleur / Examining doctor: FERNANDO GANCHEGUI (nom/name) (signature)
21. Inspecteur antidopage / Antidoping inspector: VANDEVYVERE MARC (BEL) COMMIS. INT. U.C.I. INSP. ANTIDOPAGE (nom/name) (signature)

USADA 0626

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL COMITÉ OLÍMPICO INTERNACIONAL Y AGENCIA MUNDIAL ANTIDOPAJE

PROCÈS-VERBAL: 041219 **DONNÉES GÉNÉRALES**

CONTRÔLE: EN COMPETICIÓN		**Organisme expéditeur:** UCI	
Sport: CICLISMO	**Modalité Sportive:** RUTA		
Journée, étape ou match: 8ª ETAPA	**Lieu du contrôle:** ALMUSAFES	**Date de la prise d'échantillons:** 11/09/2004	**Date de réception:** 13/09/2004
Compétition, épreuve ou activité sportive: VUELTA CICLISTA A ESPAÑA		**Date début d'analyse:** 13/09/2004	**Date fin d'analyse:** 15/09/2004

Remarques:

PROCÈS-VERBAL: 041219 **DONNÉES DES ÉCHANTILLONS "A"**

Registre du Laboratoire	Code du cachet	Code du flacon "A"
04127068A	CSD 342856	CSD A342856

PROCÈS-VERBAL: 041219 **RÉSULTATS DES ANALYSES**

Dans l'échantillon d'urine signalé ci-dessus, analysé conformément aux procédés: PNT-DE-01, PNT-DE-02, PNT-DE-03, PNT-DE-05, PNT-DE-07, PNT-CO-07 et quantification de bCG, on a détecté et confirmé:

- TRIAMCINOLONE ACETONIDE

Il est déclaré que les résultats obtenus ne concernent que les échantillons ayant été soumis à l'analyse.

Madrid, le 16-sep-04

Le Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES
LABORATORIO CONTROL DOPAJE MADRID

Dr. Agustín F. Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinfrodriguez@csd.mec.es

USADA 0627

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

ENAC
N°. 27/LE508

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL C.I.O / W.A.D.A.

PROCÈS-VERBAL: 041219 **RAPPORT T/E**

Enregistrement du laboratoire	Code du flacon "A"	T/E	Procédé
04127068A	CSD A342856	2,0 ✓	PNT-DE-03

Madrid, le 16-sep-04
Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES
LABORATORIO CONTROL DOPAJE
MADRID

Dr. Agustín F. Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinf.rodriguez@csd.mec.es

GRECO S/N
28040 MADRID
TEL: 915 696 889
FAX: 915 437 290

USADA 0628

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☒
 Hors compétition / Out of competition: ☐
2. Sexe: Masculin / Male ☒
 Sex: Féminin / Female ☐
3. Date: 12.9.2004
4. Lieu / Place: ALTO DE AITANA (ESP)
5. Course (nom, étape): 9ème étape - XÁTIVA - ALTO DE AITANA
 Race (name, stage): VUELTA A ESPAÑA
6. Discipline: ROUTE
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD
 First name:
 Adresse du coureur: BISHOP ROAD 23356 CA 92562 MURRIETA (USA)
 Address of the rider:
8. Code UCI: USA 19750174
 UCI Code:
9. Fédération nationale ayant délivré la licence: U.S.A
 National federation which delivered the license:
10. Numéro de licence: B 0020272
 License number:
11. Tiré au sort: Oui / Yes ☐
 Random: Non / No ☒
12. Heure de présentation: 17 h 35'
 Presentation time:
13. Heure de prélèvement: 17 h 43'
 Time of sampling:
14. Code des flacons: 342850 A+B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris:
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé:
Contents of the health booklet: 26-01-2004 Seretide (Asma bronquial) 8-04-2004 Positon crema (Traumatología AC) Dermatología y mucho 22-04-2004 Betametasona (Tendinitis rodilla dcha) 9-09-2004 Traumatología (Tendinitis rodilla).

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☒
 Therapeutic use exemption (TUE) Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur:
 Assistant: (nom/name) (signature)
20. Médecin contrôleur: FERNANDO GONZALEZ
 Examining doctor: (nom/name) (signature)
21. Inspecteur antidopage: VANDEVYVERE MARC (BEL) COMMIS. INT. U.C.I. ANTIDOPAGE ET MEDICAL
 Antidoping inspector: (nom/name) (signature)

USADA 0629

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL COMITÉ OLÍMPICO INTERNACIONAL Y AGENCIA MUNDIAL ANTIDOPAJE

PROCÈS-VERBAL: 041220 **DONNÉES GÉNÉRALES**

CONTRÔLE: EN COMPETICIÓN		**Organisme expéditeur:** UCI	
Sport: CICLISMO	**Modalité Sportive:** RUTA		
Journée, étape ou match: 9ª ETAPA	**Lieu du contrôle:** ALTO DE AITANO	**Date de la prise d'échantillons:** 12/09/2004	**Date de réception:** 13/09/2004
Compétition, épreuve ou activité sportive: VUELTA CICLISTA A ESPAÑA		**Date début d'analyse:** 13/09/2004	**Date fin d'analyse:** 15/09/2004

Remarques:

PROCÈS-VERBAL: 041220 **DONNÉES DES ÉCHANTILLONS "A"**

Registre du Laboratoire	Code du cachet	Code du flacon "A"
04127073A	CSD 342850	CSD A342850

PROCÈS-VERBAL: 041220 **RÉSULTATS DES ANALYSES**

Dans l'échantillon d'urine signalé ci-dessus, analysé conformément aux procédés: PNT-DE-01, PNT-DE-02, PNT-DE-03, PNT-DE-05, PNT-DE-07, PNT-CO-07 et quantification de hCG, on a détecté et confirmé:

- TRIAMCINOLONE ACETONIDE

Il est déclaré que les résultats obtenus ne concernent que les échantillons ayant été soumis à l'analyse.

Madrid, le 16-sep-04

Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES
LABORATORIO CONTROL DOPAJE MADRID

Dr. Agustín F. Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinf.rodriguez@csd.mec.es

GRECO S/N
28040 MADRID
TEL: 915 896 889
FAX: 915 437 290

USADA 0630

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

ENAC
Nº 27/LE505

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL C.I.O / W.A.D.A.

PROCÈS-VERBAL: 041220 **RAPPORT T/E**

Enregistrement du laboratoire	Code du flacon "A"	T/E	Procédé
04127073A	CSD A342850	1,4 ✓	PNT-DE-03

Il est déclaré que les résultats obtenus ne concernent que les échantillons ayant été soumis à l'analyse.

Madrid, le 16-scp-04
Le Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES
LABORATORIO CONTROL DOPAJE MADRID

Dr. Agustín F. Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinf.rodriguez@csd.mec.es

GRECO S/N
28040 MADRID
TEL: 915 896 889
FAX: 915 437 290

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☒
 Hors compétition / Out of competition: ☐
2. Sexe: Masculin / Male ☒
 Sex: Féminin / Female ☐
3. Date: 13.9.2004
4. Lieu / Place: XORRET DE CATI (ESP)
5. Course (nom, étape): VUELTA A ESPAÑA
 Race (name, stage):
6. Discipline: ROUTE
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD
 First name:
 Adresse du coureur: Bishop Road 23356 CA 92562 MURRIETA (USA)
 Address of the rider:
8. Code UCI: USA19750714
 UCI Code:
9. Fédération nationale ayant délivré la licence: U.S.A.
 National federation which delivered the license:
10. Numéro de licence: B00202 72
 License number:
11. Tiré au sort: Oui / Yes ☐
 Random: Non / No ☒
12. Heure de présentation: 17 h 45'
 Presentation time:
13. Heure de prélèvement: 17 h 50'
 Time of sampling:
14. Code des flacons: 348369 A+B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris:
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé:
Contents of the health booklet: 26-01-2004 Seretide (Asma bronquial)
8-04-2004 Triamcinolona Ac. (Dermatitis pie y mano izq)
22-04-2004 Betametasona (Tendinitis rodilla derecha)
9-09-2004 Triamcinolona (Tendinitis rodilla derecha)

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☒
 Therapeutic use exemption (TUE) Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur:
 Assistant: (nom/name) (signature)
20. Médecin contrôleur: FERNANDO GANCHEGUI
 Examining doctor: (nom/name) (signature)
21. Inspecteur antidopage: VANDEVYVERE MARC (BEL)
 Antidoping inspector: COMMIS. INT. U.C.I. (signature)
 INSP. ANTIDOPAGE FT MEDICAL
 (nom/name) (signature)

USADA 0632

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL COMITÉ OLÍMPICO INTERNACIONAL Y AGENCIA MUNDIAL ANTIDOPAJE

PROCÈS-VERBAL: 041223 **DONNÉES GÉNÉRALES**

CONTRÔLE: EN COMPETICIÓN		**Organisme expéditeur:** UCI	
Sport: CICLISMO	**Modalité Sportive:** RUTA		
Journée, étape ou match: 10ª ETAPA	**Lieu du contrôle:** XORRET	**Date de la prise d'échantillons:** 13/09/2004	**Date de réception:** 14/09/2004
Compétition, épreuve ou activité sportive: VUELTA CICLISTA A ESPAÑA		**Date début d'analyse:** 14/09/2004	**Date fin d'analyse:** 16/09/2004

Remarques:

PROCÈS-VERBAL: 041223 **DONNÉES DES ÉCHANTILLONS "A"**

Registre du Laboratoire	Code du cachet	Code du flacon "A"
04127091A	348369	A348369

PROCÈS-VERBAL: 041223 **RÉSULTATS DES ANALYSES**

Les échantillons d'urine signalés ci-dessus ont été analysés dans ce Laboratoire conformément aux procédés: PNT-DE-01, PNT-DE-02, PNT-DE-03, PNT-DE-05, PNT-DE-07 et quantification de hCG sans qu'aucune substance pouvant être à l'origine d'un résultat positif de contrôle du dopage n'y ait été détectée, que ce soit qualitativement ou quantitativement.

Il est déclaré que les résultats obtenus ne concernent que les échantillons ayant été soumis à l'analyse.

Madrid, le 16-sep-04

Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES LABORATORIO MADRID

Dr. Agustín Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinf.rodriguez@csd.mec.es

USADA 0633

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL C.I.O / W.A.D.A.

ENAC
LABORATORIOS
Nº 27/LE506

PROCÈS-VERBAL: 041223 **RAPPORT T/E**

Enregistrement du laboratoire	Code du flacon "A"	T/E	Procédé
04127091A	A348369	1,3 ✓	PNT-DE-03

Il est déclaré que les résultats obtenus ne concernent que les échantillons ayant été soumis à l'analyse.

Madrid, le 16-sep-04

Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES
LABORATORIO CONTROL DOPAJE
MADRID

Dr. Agustín F. Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinf.rodriguez@csd.mec.es

USADA 0634

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☒
 Hors compétition / Out of competition: ☐
2. Sexe / Sex: Masculin / Male ☒ — Féminin / Female ☐
3. Date: 14.9.2004
4. Lieu / Place: CARAVACA DE LA CRUZ
5. Course (nom, étape) / Race (name, stage): VUELTA A ESPAÑA — 14ème étape: J. VICENTE DE ARAGÜETE - CARAVACA
6. Discipline / Discipline: ROUTE
7. Nom du coureur / Surname of rider: ARMSTRONG — Prénom / First name: FLOYD
 Adresse du coureur / Address of the rider: BISHOP RD 23356 CA92562 MURRIETA (USA)
8. Code UCI / UCI Code: USA19751014
9. Fédération nationale ayant délivré la licence / National federation which delivered the license: U.S.A
10. Numéro de licence / License number: 3002072
11. Tiré au sort / Random: Oui / Yes ☐ — Non / No ☒
12. Heure de présentation / Presentation time: 17 h 40'
13. Heure de prélèvement / Time of sampling: 17 h 45'
14. Code des flacons / Bottle codes: 347883 A+B
15. Heure de refus du coureur / Time of rider's refusal:
16. Médicaments pris / Pharmaceutical drugs taken by rider:

Contenu du livret de santé / Contents of the health booklet:
26-01-2004 Seretaid (Asma bronquial)
8-04-2004 Triamcinolona (Dermatitis pies y manos)
22-04-2004 Betametasona (Tendinitis rodilla dcha)
9-9-2004 Triamcinolona (Tendinitis rodilla dch)

17. Autorisation d'usage à des fins thérapeutiques (AUT) / Therapeutic use exemption (TUE): Oui / Yes ☒ — Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement. / Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie / Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur / Assistant: (nom/name) (signature)
20. Médecin contrôleur / Examining doctor: FERNANDO GANCHEGUI (nom/name) (signature)
21. Inspecteur antidopage / Antidoping inspector: VANDEVYVERE MARC (nom/name) COMMIS. INT. U.C.I. ~~INSP. ANTIDOPAGE ET MEDICAL~~ (signature)

USADA 0635

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL COMITÉ OLÍMPICO INTERNACIONAL Y AGENCIA MUNDIAL ANTIDOPAJE

PROCÈS-VERBAL: 041231 **DONNÉES GÉNÉRALES**

CONTRÔLE: EN COMPETICIÓN		**Organisme expéditeur:** UCI	
Sport: CICLISMO	**Modalité Sportive:** RUTA		
Journée, étape ou match: 11ª ETAPA	**Lieu du contrôle:** CARAVACA DE LA CRUZ	**Date de la prise d'échantillons:** 14/09/2004	**Date de réception:** 15/09/2004
Compétition, épreuve ou activité sportive: VUELTA CICLISTA A ESPAÑA		**Date début d'analyse:** 15/09/2004	**Date fin d'analyse:** 17/09/2004

Remarques:

PROCÈS-VERBAL: 041231 **DONNÉES DES ÉCHANTILLONS**

Registre du Laboratoire	Code du cachet	flacon "A"
04127128A	347883	A347883

PROCÈS-VERBAL: 041231 **RÉSULTATS DES ANALYSES**

Les échantillons d'urine signalés ci-dessus ont été analysés dans ce Laboratoire conformément aux procédés: PNT-DE-01, PNT-DE-02, PNT-DE-03, PNT-DE-05, PNT-DE-07 et quantification de hCG sans qu'aucune substance pouvant être à l'origine d'un résultat positif de contrôle du dopage n'y ait été détectée, que ce soit qualitativement ou quantitativement.

Il est déclaré que les résultats obtenus ne concernent que les échantillons ayant été soumis à l'analyse.

Madrid, le 17-sep-04

Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES - LABORATORIO DOPAJE - MADRID

Dr. Agustín Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinf.rodriguez@csd.mec.es

GRECO S/N
28040 MADRID
TEL: 915 896 889
FAX: 915 437 280

USADA 0636

MINISTERIO
DE EDUCACIÓN
Y CIENCIA

CONSEJO SUPERIOR DE DEPORTES

ENAC
ENSAYOS
Nº 270/LE605

LABORATORIO DE CONTROL DEL DOPAJE (MADRID)

ACREDITADO POR EL C.I.O / W.A.D.A.

PROCÈS-VERBAL: 041231 **RAPPORT T/E**

Enregistrement du laboratoire	Code du flacon "A"	T/E	Procédé
04127128A	A347883	1,0 ✓	PNT-DE-03

Il est déclaré que les résultats obtenus ne concernent que les échantillons ayant été soumis à l'analyse.

Madrid, le 17-sep-04

Directeur du Laboratoire

CONSEJO SUPERIOR DE DEPORTES
LABORATORIO CONTROL DOPAJE
MADRID

Dr. Agustín F. Rodríguez Cano

Il est notifié, à tout effet, que cet acte ne peut être reproduit partiellement sans l'approbation écrite du Laboratoire.

Correo electrónico:
agustinf.rodriguez@csd.mec.es

GRECO S/N
28040 MADRID
TEL: 915 896 88
FAX: 915 437 290

USADA 0637

UNITED STATES ANTI-DOPING AGENCY
Doping Control Official Record

Floyd Landis
ATHLETE NAME / OR PLACE / OR DISCIPLINE

1-8-05
NOTIFICATION DATE

810 AM PM
TIME

Pursuant to USADA Regulations:

- You are required to be drug tested. Signature on this form constitutes your consent to such testing.
- You have a maximum of 60 minutes from the time of notification to arrive with your Chaperone at the Doping Control Station, unless being tested under the Out-of-Competition conditions.
- You should bring a photo ID with you to the Doping Control Station.
- **Refusal to cooperate or failure to report within the given time limit may subject you to sanctions consistent with a doping violation.**
- Consumption of any fluid or food even if provided by the Chaperone or a Doping Control Officer is at your own risk.
- You may have a representative accompany you through the testing process, but such representative may not witness the voiding.

Thomas R McVay
Notifying Chaperone (Print Name)

Athlete Signature

Floyd Landis
(Print Name)

ATHLETE INFORMATION

Floyd Landis
Athlete Name (Print)

1-8-05
ARRIVAL DATE

810 AM PM
TIME

23356 Bishop Rd
Mailing Address

Murrieta CA 92562
City, State, Zip Code, COUNTRY

10-14-75
Date of Birth (month / day / year)

951 461-8462
Phone

CA DL D2203288
Photo Identification Type / No.

LABORATORY/SPECIMEN INFORMATION

☐ Event ☒ Out-of-Competition ☐ Other _____

☐ Full Menu Test ☒ Partial Menu Test

1-8-05
SAMPLE PROCESSING DATE

925 AM PM
TIME

OOC
e ID Number ("OOC" if Out-of-Competition)

Cycling
Sport

Murrieta CA
City/State, if OOC Competition Name, if Event

4851555
SAMPLE CODE NUMBER

☒ MALE ☐ FEMALE

Specific Gravity and pH levels within range ☒ YES ☐ NO

Specific Gravity: Greater than or equal to 1.005 (≥ 1.005)
pH: Not less than 5.0 and not greater than 7.5 (5.0 ≥ x ≤ 7.5)

SUBSEQUENT SPECIMEN

SUBSEQUENT SAMPLE CODE NUMBER

AM PM
TIME

Specific Gravity and pH levels within range ☐ YES ☐ NO

Declaration of medications and other substances, including vitamins, minerals, herbs and other dietary supplements, taken during the preceding three (3) days (write "none" if none declared):

Name of Substance	Dose	Date Last Taken	Name of Substance	Dose	Date Last Taken
Oxarrozin	1 dose	1-7-05			
Ambien	1 dose	1-7-05			
Vitamin	1 dose	1-7-05			
Fish oil	1 dose	1-7-05			

SIGNATURES

By signing below I certify that: (i) I selected a sealed collection container and a sample kit from the supply provided, opened and visually checked that they were empty and clean, sealed them securely and confirmed that the numbers on the sample kit and bottles were identical to the numbers written on this *Doping Control Official Record*; (ii) the Doping Control Officer did not handle my sample(s) except when permitted by me; (iii) no irregularities occurred in the sample collection process; and (iv) this information is not being provided to USADA pursuant to a doctor/patient relationship and is not to be considered a confidential medical record.

Athlete Signature — Floyd Landis (Print Name) — N/A Language Specialist Signature (or "n/a" if none) (Print Name)

The following individuals were present during the sample collection and/or processing procedure(s).

Thomas R McVay — N/A
Chaperone Signature (Print Name) — Subsequent Chaperone Signature (or "n/a" if none) (Print Name)

Thomas R McVay — N/A
Doping Control Officer Signature (Print Name) — Athlete Representative Signature (or "n/a" if none) (Print Name)

☐ Partial Sample Form ☐ Supplementary Report Form

©USADA

WHITE = Master BLUE = USADA YELLOW = Laboratory PINK = Athlete

IOC Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave. Los Angeles, CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ACCREDITED
ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL

Attached is the report USADA6680
and the corresponding pH and specific gravity report.

regards
UCLA Olympic Analytical Laboratory

Yulia Kucherova

Wednesday, January 19, 2005

USADA6680

UCLA,USADA6680,"OOC ","These NEGATIVE urine samples were analyzed for anabolic and masking agents, diuretics, and hCG using methods 1001, 2001, 6001, and LCMSMS."

4JF04,A485155,01082005,01082005,"CYCLING",T,T,F

4JF04,A485155,7.5.1.012

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☐
 Hors compétition / Out of competition: ☐
2. Sexe: Masculin / Male ☑
 Sex: Féminin / Female ☐
3. Date: APRIL 21, 2005
4. Lieu / Place: ROME GA
5. Course (nom, étape):
 Race (name, stage): TOUR DE GEORGIA
6. Discipline: ROAD T.T.
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD.
 First name:
 Adresse du coureur: 23356 MERIETA CA 92562.
 Address of the rider: BISHOP RD.
8. Code UCI: USA19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA
 National federation which delivered the license:
10. Numéro de licence: 0020292.
 License number:
11. Tiré au sort: Oui / Yes ☐
 Random: Non / No ☑
12. Heure de présentation: 1450
 Presentation time:
13. Heure de prélèvement: 1455.
 Time of sampling:
14. Code des flacons: 920460 A+B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris:
 Pharmaceutical drugs taken by rider: None

Contenu du livret de santé:
Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☑
 Therapeutic use exemption (TUE) Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur:
 Assistant: (nom/name) (signature)
20. Médecin contrôleur: ROBERT T. PUCKETT, MD
 Examining doctor: (nom/name) (signature)
21. Inspecteur antidopage: BILL LAMBART Bill Lambart
 Antidoping inspector: (nom/name) (signature)

USADA 0642

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: May 2, 2005

3. Date of receipt of the sample: April 22, 2005 time: 10:03 A.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 04/21/05
Race (name,stage): Tour de Georgia
Place: Georgia, USA
Discipline: Road

5. Identification of the sample examined:

- UCI code number: **920460** = UCLA # **5FP12**
- external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

- appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.015**

7. Date of analysis: April 22, 2005 - May 2, 2005

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

The following substances have been found in the sample (quantity or concentration are indicated, if applicable):

NONE.

10. Enclosures: CIRCLE ONE NUMBER BELOW

1. report of the analysis
2. chromatogram (indicate "none" when applicable)
3. mass spectrogram (indicate "none" when applicable)
(4) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Yulia Kucherova

Certifying Scientist

Signature:

HEURE DE RECEPTION 3. MAI. 0:05

HEURE D'IMPRESSION 3. MAI 0:08

CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

Contrôle urinaire / Urine test ☑

~~Contrôle sanguin / Blood test~~ ☐

1. En compétition / In competition ☑
 Hors compétition / Out of competition ☐

2. Sexe: Masculin / Male ☑
 Sex: Féminin / Female ☐

3. Date: APRIL 22 2005

4. Lieu / Place: DAHLONEGA GA.

5. Course (nom, étape):
 Race (name, stage): TOUR DE GEORGIA

6. Discipline: ROAD
 Discipline:

7. Nom du coureur: LANDIS
 Surname of rider:

 Prénom: FLOYD.
 First name:

 Adresse du coureur: 23356 BISHOP RD. MURRIETA. CA. 92562.
 Address of the rider:

8. Code UCI: USA19751014.
 UCI Code:

9. Fédération nationale ayant délivré la licence: USA.
 National federation which delivered the license:

10. Numéro de licence: 0020172.
 License number:

11. Tiré au sort / Random: Oui / Yes ☐ Non / No ☑

12. Heure de présentation: 1500
 Presentation time:

 Volume _____ pH* _____
 *Optionnel / Optional

13. Heure de prélèvement: 1510.
 Time of sampling:

 Densité / Specific gravity* _____

14. Code des flacons: 920462 A+B
 Bottle codes:

15. Heure de refus du coureur:
 Time of rider's refusal:

16. Médicaments pris: NONE
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé: NONE
Contents of the ~~health booklet~~:

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☐
 Therapeutic use exemption (TUE) Non / No ☐

18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur:
 Assistant: (nom/name) (signature)

20. Médecin contrôleur: Richard A. Wheatmo
 Examining doctor: (nom/name) (signature)

 Agent de prélèvement sanguin:
 Blood collection officer: (nom/name) (signature)

21. Inspecteur antidopage: Bill LAMBART Bill Lambart
 Antidoping inspector: (nom/name) (signature)

USADA 0644

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: May 4, 2005

3. Date of receipt of the sample: April 25, 2005 time: 9:55 A.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 04/22/05
Race (name,stage): Tour de Georgia
Place: Georgia, USA
Discipline: Road

5. Identification of the sample examined:

- UCI code number: **920462** = UCLA # **5HF05**
- external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

- appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.025**

7. Date of analysis: April 25, 2005 - May 4, 2005

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

The following substances have been found in the sample (quantity or concentration are indicated, if applicable):

NONE.

10. Enclosures: CIRCLE ONE NUMBER BELOW

1. report of the analysis
2. chromatogram (indicate "none" when applicable)
3. mass spectrogram (indicate "none" when applicable)
(4) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Yulia Kucherova — Signature:

Certifying Scientist

HEURE DE RECEPTION 5. MAI. 0:29 HEURE D'IMPRESSION 5. MAI. 0:32

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS, FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : U.S.A.

Adresse / Postal adress : 23356 BISHOP ROAD MURRIERA CALIFORNIA U.S.A.

Organisme demandant le contrôle / Organization requesting the test :
- ☒ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 002027 2 / USA 19751014

Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : U.S.A. Date de naissance / Birth Date : 14.10.1975 Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : F.F. DE CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : T. DE F. 15-IEME ETAPE

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 17-07-2005 Heure de remise de la notification : 16.50 h Lieu du contrôle / Place of test : L'AUTOBUS CONTR. A.D.

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested LANDIS FLOYD

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : JAN VAN CHESTEL

Nom et prénom du médecin / Name of doctor (en toutes lettres) : HUBERT LONG

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17.30 h Date du prélèvement / Sampling date : 17-07-2005 Heure du prélèvement / Sampling time : 17.40 h

Identification / Identification : Licence / Licence : ☒ Carte d'identité / ID : ☐ Passeport / Passport : ☐ Autre / Other : ☒

Échantillons A et B / Samples A and B — Code Flacon (et container) n° / bottle Code : 874535

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 75 cc

Densité / Specific Gravity : 1.025 pH / pH : 6.0

Échantillon d'urine insuffisant / Insufficient urine sample

cellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non — Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)

1.) AUT Betamethasone inj (28.05.05)

2.) AUT ~~Betamethasone~~ Glucocorticoïdes 2 injs de 6 ml 1 puis la dernière semaine

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

USADA 0646

IMPRIMERIE NATIONALE 4000001

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 22 juillet 2005

25814

RAPPORT D'ANALYSE N° 85/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr LONG

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2005 (15ème étape) à St Lary Soulan**
Date :	**17/07/2005**

Réception de(s) l'échantillon(s) d'urine :

Date :	**17/07/2005**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**3 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**4**
Référence de(s) l'échantillon(s) :	**85/07_874535**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **18/07/2005**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES01, ES02, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : **Recherche de substances dopantes négative**

NB1 : Echantillon n° 874535 : Les résultats des essais ES04 et ES05 ne sont pas totalement exploitable en raison d'une inhibition de la dérivation due à une interférence matricielle.

J. de CEAURRIZ
Directeur

Destinataires :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°85/07 et 85/07-00

Résultats des rapports T/E

Référence des échantillons	T/E
874535	2.2

J. de CHAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

UNITED STATES ANTI-DOPING AGENCY
Doping Control Official Record

Floyd Landis — 10-7-05 — 6:45 AM/PM

P Name Required (Place and/or Discipline if In-Competition) — Notification Date — Time

I ant to USADA Regulations:

- You are required to be drug tested. Signature on this form constitutes your consent to such testing.
- You have a maximum of 60 minutes from the time of notification to arrive with your Chaperone at the Doping Control Station, unless being tested under the Out-of-Competition conditions.
- You should bring a photo ID with you to the Doping Control Station.
- **Refusal to cooperate or failure to report within the given time limit may subject you to sanctions consistent with an anti-doping rule violation.**
- Consumption of any fluid or food even if provided by the Chaperone or a Doping Control Officer is at your own risk.
- You may have a Representative accompany you through the testing process, but such Representative may not witness the voiding.

KRIS FORBERG — Notifying Chaperone (Print Name)

Floyd Landis — Athlete Signature (Print Name)

ATHLETE INFORMATION

23356 Bishop Rd. — Mailing Address

Murrieta, CA. 92562 USA — City, State, Zip Code, COUNTRY

951-461-8462 — Phone

10-7-05 — Arrival Date

6:45 AM/PM — Time

10-14-1975 — Date of Birth (month / day / year)

CALIFORNIA DRIVERS LIC. D2263288 — Photo Identification Type / No.

LABORATORY/SAMPLE INFORMATION

☐ In-Competition ☒ Out-of-Competition ☐ Other

☐ Full Menu Test ☒ Partial Menu Test

Test Conducted For: n/a (or "n/a" if USADA)

OOC — Site ID Number ("OOC" if Out-of-Competition)

Xcling — Sport — Discipline (if available)

Murrieta, CA. — City/State, if OOC — Competition Name, if Event

10-7-05 — Sample Processing Date

6:52 AM/PM — Time

4	9	1	6	0	7

SAMPLE CODE NUMBER

☒ MALE ☐ FEMALE

Specific Gravity and pH levels within range? ☒ YES ☐ NO

Specific Gravity: Greater than or equal to 1.005 (≥ 1.005)

pH: Not less than 5.0 and not greater than 7.5 (5.0 > x < 7.5)

SUBSEQUENT SAMPLE

SUBSEQUENT SAMPLE CODE NUMBER — Time AM/PM

Specific Gravity and pH levels within range? ☐ YES ☐ NO

Declaration of any recent blood transfusions, as well as any medications and other substances, including vitamins, minerals, herbs and other dietary supplements, taken during the preceding three (3) days (write "none" if none declared and draw a line through any unused spaces):

Name of Substance	Dose	Date Last Taken
Multivitamin	2 pills	10-6-05
Glucosamine HCl/ CHONDROITIN	2 pills	10-6-05
IRON 325 mg	1 pill	10-6-05
FISH OIL	3 pills	10-6-05

Name of Substance	Dose	Date Last Taken

Consent for research (optional): By signing below I agree that this Sample may be used for anti-doping research purposes. When all analyses have been completed, and this Sample would otherwise be discarded, it may then be used by any WADA approved laboratory for anti-doping research of any type, provided that it can no longer be identified as my Sample.

☑ I Accept ☐ I Decline

Signature:

SIGNATURES

By signing below I certify that: (i) I selected a sealed collection container and a Sample kit from the supply provided, opened and visually checked that they were empty and clean, sealed them securely and confirmed that the numbers on the Sample kit and bottles were identical to the numbers written on this *Doping Control Official Record*; (ii) the Doping Control Officer did not handle my Sample(s) except when permitted by me; (iii) no irregularities occurred in the Sample Collection Procedure; and (iv) this information is not being provided to USADA pursuant to a doctor/patient relationship and is not to be considered a confidential medical record.

Athlete Signature — Floyd Landis (Print Name)

The following individuals were present during the Sample Collection Session.

Chaperone Signature — KRIS FORBERG (Print Name)

n/a — Language Specialist Signature (or "n/a" if none) (Print Name)

n/a — Subsequent Chaperone Signature (or "n/a" if none) (Print Name)

n/a — Athlete Representative Signature (or "n/a" if none) (Print Name)

Doping Control Officer Signature — KRIS FORBERG (Print Name)

n/a — Other Signature (or "n/a" if none)

☐ Partial Sample Form ☐ Laboratory Report Form

IOC Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave. Los Angeles, CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ACCREDITED
ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL

Attached is the report USADA7302
and the corresponding pH and specific gravity report.

regards
UCLA Olympic Analytical Laboratory

Yulia Kucherova

Tuesday, October 25, 2005

USADA7302

UCLA,USADA7302,"OOC ","These NEGATIVE urine samples were analyzed for anabolic and masking agents, diuretics, and hCG using methods 1001, 2001, and, 6001."
63L03,A491607,10072005,10082005,"CYCLING",T,T,F

63L03,A491607,5.0,1.026

UNITED STATES ANTI-DOPING AGENCY
Doping Control Official Record

Floyd Landis — 11-5-05 — 2:40 AM (PM)

e Name Required (Place and/or Discipline if In-Competition) — Notification Date — Time

uant to USADA Regulations:

- You are required to be drug tested. Signature on this form constitutes your consent to such testing.
- You have a maximum of 60 minutes from the time of notification to arrive with your Chaperone at the Doping Control Station, unless being tested under the Out-of-Competition conditions.
- You should bring a photo ID with you to the Doping Control Station.
- **Refusal to cooperate or failure to report within the given time limit may subject you to sanctions consistent with an anti-doping rule violation.**
- Consumption of any fluid or food even if provided by the Chaperone or a Doping Control Officer is at your own risk.
- You may have a Representative accompany you through the testing process, but such Representative may not witness the voiding.

KRIS FORBERG — Floyd Landis

Notifying Chaperone (Print Name) — Athlete Signature — (Print Name)

ATHLETE INFORMATION

23356 Bishop Rd. — 11-5-05 — 2:40 AM (PM)

Mailing Address — Arrival Date — Time

Murrieta, CA. 92562 — 10-14-1975

City, State, Zip Code, COUNTRY — Date of Birth (month / day / year)

951-461-8462 — CALIFORNIA DRIVERS LIC. — D2203288

Phone — Photo Identification Type — No.

LABORATORY/SAMPLE INFORMATION

☐ In-Competition ☒ Out-of-Competition ☐ Other

☐ Full Menu Test ☒ Partial Menu Test

Test Conducted For: n/a (or "n/a" if USADA)

11-5-05 — 3:04 AM (PM)

Sample Processing Date — Time

OOC

Site ID Number ("OOC" if Out-of-Competition)

4 9 3 0 8 4 — ☒ MALE ☐ FEMALE

SAMPLE CODE NUMBER

Specific Gravity and pH levels within range? ☒ YES ☐ NO

Specific Gravity: Greater than or equal to 1.005 (≥ 1.005)

pH: Not less than 5.0 and not greater than 7.5 (5.0 ≥ x < 7.5)

Cycling

Sport — Discipline (if available)

Murrieta, CA

City/State, if OOC — Competition Name, if Event

SUBSEQUENT SAMPLE

SUBSEQUENT SAMPLE CODE NUMBER — AM PM Time

Specific Gravity and pH levels within range? ☐ YES ☐ NO

Declaration of any recent blood transfusions, as well as any medications and other substances, including vitamins, minerals, herbs and other dietary supplements, taken during the preceding three (3) days (write "none" if none declared and draw a line through any unused spaces):

Name of Substance	Dose	Date Last Taken
FISH OIL	3 pills	11-5-05
Glucosamine HCl 1500mg/Chondroitin	2 pills	11-5-05
Multivitamin	2 pills	11-5-05
VIT. E	2 pills	11-4-05

Name of Substance	Dose	Date Last Taken

Consent for research (optional): By signing below I agree that this Sample may be used for anti-doping research purposes. When all analyses have been completed, and this Sample would otherwise be discarded, it may then be used by any WADA approved laboratory for anti-doping research of any type, provided that it can no longer be identified as my Sample. ☑ I Accept ☐ I Decline

Signature:

SIGNATURES

By signing below I certify that: (i) I selected a sealed collection container and a Sample kit from the supply provided, opened and visually checked that they were empty and clean, sealed them securely and confirmed that the numbers on the Sample kit and bottles were identical to the numbers written on this *Doping Control Official Record*; (ii) the Doping Control Officer did not handle my Sample(s) except when permitted by me; (iii) no irregularities occurred in the Sample Collection Procedure; and (iv) this information is not being provided to USADA pursuant to a doctor/patient relationship and is not to be considered a confidential medical record.

Floyd Landis

Athlete Signature — (Print Name)

The following individuals were present during the Sample Collection Session.

Kris Forberg — KRIS FORBERG

Chaperone Signature — (Print Name)

n/a

Language Specialist Signature (or "n/a" if none) — (Print Name)

n/a — (Print Name)

Subsequent Chaperone Signature (or "n/a" if none)

Kris Forberg — KRIS FORBERG

Doping Control Officer Signature — (Print Name)

n/a — (Print Name)

Athlete Representative Signature (or "n/a" if none)

n/a

Other Signature (or "n/a" if none)

IOC Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave. Los Angeles, CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ACCREDITED
ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL

Attached is the report USADA7361
and the corresponding pH and specific gravity report.

regards
UCLA Olympic Analytical Laboratory

Yulia Kucherova

Tuesday, November 22, 2005

USADA7361

UCLA,USADA7361,"OOC ","These NEGATIVE urine samples were analyzed for anabolic and masking agents, diuretics, and hCG using methods 1001, 2001, and, 6001."
6W903,A493084,11052005,11082005,"CYCLING",T,T,F

6W903,A493084.5.0.1.018

USADA UNITED STATES ANTI-DOPING AGENCY
Doping Control Official Record

Floyd Landis — 1/6/06 — 7:04 AM / PM

ete Name Required (Place and/or Discipline if In-Competition) — Notification Date — Time

suant to USADA Regulations:

- You are required to be drug tested. Signature on this form constitutes your consent to such testing.
- **Refusal to cooperate or failure to comply with the doping control process will subject you to at least a 2-year suspension and other sanctions consistent with an anti-doping rule violation.**
- You have a maximum of 60 minutes from the time of notification to arrive with your Chaperone at the Doping Control Station, unless being tested under the Out-of-Competition conditions.
- You should bring a photo ID with you to the Doping Control Station.
- Consumption of any fluid or food (even if provided by the Chaperone or a Doping Control Officer) is at your own risk.
- You may have a Representative accompany you through the testing process, but such Representative may not witness the sample collection.

KRIS FORBERG — Notifying Chaperone (Print Name)

Athlete Signature

Floyd Landis — (Print Name)

ATHLETE INFORMATION

23756 Bishop Road — Mailing Address

Murrieta CA 92562 — City, State, Zip Code, COUNTRY

951 461 8962 — Phone

1/6/06 — Arrival Date

7:04 AM / PM — Time

10/14/75 — Date of Birth (month / day / year)

Drivers License CA D2203288 — Photo Identification Type / No.

LABORATORY/SAMPLE INFORMATION

☐ In-Competition ☒ Out-of-Competition ☐ Other _____

☐ Full Menu Test ☒ Partial Menu Test

Test Conducted For: n/a (or "n/a" if USADA)

OOC — Site ID Number ("OOC" if Out-of-Competition)

Cycling — Sport

Discipline (if available)

Murrieta — City/State (if OOC)

CA. — Event Name (if In-Competition)

1/6/06 — Sample Processing Date

7:18 AM / PM — Time

4	9	3	0	9	1	✓

SAMPLE CODE NUMBER

☒ MALE ☐ FEMALE

Specific Gravity and pH levels within range? ☒ YES ☐ NO

Specific Gravity: Greater than or equal to 1.005 (≥ 1.005)

pH: Not less than 5.0 and not greater than 7.5 (5.0 ≥ x ≤ 7.5)

SUBSEQUENT SAMPLE

SUBSEQUENT SAMPLE CODE NUMBER

_____ AM / PM — Time

Specific Gravity and pH levels within range? ☐ YES ☐ NO

Declaration of any recent blood transfusions, as well as any medications and other substances, including vitamins, minerals, herbs and other dietary supplements, taken during the preceding three (3) days (write "none" if none declared and draw a line through any unused spaces):

Name of Substance	Dose	Date Last Taken
Fish Oil	2 pills	1/6/06
Glucosomine	1 pill	1/6/06

Name of Substance	Dose	Date Last Taken

Consent for research (optional): By checking "I Accept" and signing in the space provided I agree that this Sample may be used for anti-doping research purposes. When all analyses have been completed, and this Sample would otherwise be discarded, it may then be used by any WADA approved laboratory for anti-doping research of any type, provided that it can no longer be identified as my Sample.

☑ I Accept ☐ I Decline

Signature:

SIGNATURES

By signing below I certify that: (i) I selected a sealed collection container and a Sample kit from the supply provided, opened and visually checked that they were empty and clean, sealed them securely and confirmed that the numbers on the Sample kit and bottles were identical to the numbers written on this *Doping Control Official Record*; (ii) the Doping Control Officer did not handle my Sample(s) except when permitted by me; (iii) no irregularities occurred in the Sample Collection Procedure; and (iv) this information is not being provided to USADA pursuant to a doctor/patient relationship and is not to be considered a confidential medical record.

Athlete Signature — Floyd Landis (Print Name)

The following individuals were present during the Sample Collection Session.

Kris Forberg — KRIS FORBERG (Print Name)
Chaperone Signature

n/a — Language Specialist Signature (or "n/a" if none) — (Print Name)

n/a — Subsequent Chaperone Signature (or "n/a" if none) — (Print Name)

n/a — Athlete Representative Signature (or "n/a" if none) — (Print Name)

Kris Forberg — KRIS FORBERG (Print Name)
Doping Control Officer Signature

n/a — Other Signature (or "n/a" if none)

☐ Partial Sample Form ☐ Supplementary Report Form

USADA 0657

IOC Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave. Los Angeles, CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ACCREDITED
ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL

Attached is the report USADA7461
and the corresponding pH and specific gravity report.

regards
UCLA Olympic Analytical Laboratory

Friday, January 20, 2006

Yulia Kucherova

This report shall not be reproduced, except in full, without written approval of the laboratory.

USADA7461

UCLA,USADA7461,"OOC ","These NEGATIVE urine samples were analyzed for anabolic and masking agents, diuretics, and hCG using methods 1001, 2001, and 6001."

ZYG09,A493091,01062006,01072006,"CYCLING",T,T,F

7YG09	A493091	5.5	1.025

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☐
 Hors compétition / Out of competition: ☐
2. Sexe / Sex: Masculin / Male ☐ Féminin / Female ☐
3. Date: FEB 22, 2006
4. Lieu / Place: CALIFORNIA, USA
5. Course (nom, étape) / Race (name, stage): AMGEN TOUR.
6. Discipline / Discipline: ROAD
7. Nom du coureur / Surname of rider: LANDIS
 Prénom / First name: FLOYD
 Adresse du coureur / Address of the rider: 23356 Bishop Road, Murrieta CA 92562
8. Code UCI / UCI Code: USA 19751014
9. Fédération nationale ayant délivré la licence / National federation which delivered the license: USA Cycling
10. Numéro de licence / License number: 0020272
11. Tiré au sort / Random: Oui / Yes ☑ Non / No ☐
12. Heure de présentation / Presentation time: 1445
13. Heure de prélèvement / Time of sampling: 1500
14. Code des flacons / Bottle codes: 951826 A+B
15. Heure de refus du coureur / Time of rider's refusal:
16. Médicaments pris / Pharmaceutical drugs taken by rider: None

Contenu du livret de santé / Contents of the health booklet: 6

17. Autorisation d'usage à des fins thérapeutiques (AUT) / Therapeutic use exemption (TUE) Oui / Yes ☐ Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement. / Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie / Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur / Assistant: (nom/name) (signature)
20. Médecin contrôleur / Examining doctor: HOWARD E MICHAELS MD (nom/name) Howard E. Michaels MD (signature)
21. Inspecteur antidopage / Antidoping inspector: WILLIAM LAMBART (nom/name) William Lambart (signature)

USADA 0661

TO: Mr. Christian V[illegible]

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY
 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: March 13, 2006

3. Date of receipt of the sample: February 27, 2006 time: 10:10 A.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 02/22/06
Race (name,stage): Amgen Tour
Place: California, USA
Discipline: Road

5. Identification of the sample examined:

 - UCI code number: **951826** Code: **7Y303**
 - external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

 - appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.026**

7. Date of analysis: February 27, 2006 - March 13, 2006

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

 The following substances have been found in the sample
 (quantity or concentration are indicated, if applicable):

 NONE.

10. Enclosures: CIRCLE ONE NUMBER BELOW

 1. report of the analysis
 2. chromatogram (indicate "none" when applicable)
 3. mass spectrogram (indicate "none" when applicable)
 (4) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Michael Sekera — Signature:

Certifying Scientist

HEURE DE RECEPTION 14. MAR. 17:13 HEURE D'IMPRESSION 14. MAR. 17:16

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☑
 Hors compétition / Out of competition: ☐
2. Sexe: Masculin / Male ☑
 Sex: Féminin / Female ☐
3. Date: FEB 23 2006
4. Lieu / Place: CALIFORNIA USA
5. Course (nom, étape): AMGEN TOUR
 Race (name, stage):
6. Discipline: ROAD
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD
 First name:
 Adresse du coureur: 23356 BISHOP ROAD MURRIETA CA 92562
 Address of the rider:
8. Code UCI: USA 19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA CYCLING
 National federation which delivered the license:
10. Numéro de licence: 0020272
 License number:
11. Tiré au sort: Oui / Yes ☑
 Random: Non / No ☑
12. Heure de présentation: 1555
 Presentation time:
13. Heure de prélèvement: 16.00
 Time of sampling:
14. Code des flacons: 95187 5 A + B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris: NONE
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé:
Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☐
 Therapeutic use exemption (TUE) Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur:
 Assistant: (nom/name) (signature)
20. Médecin contrôleur: SCOTT A. MORE JR. MD
 Examining doctor: (nom/name) (signature) MD.
21. Inspecteur antidopage: WILLIAM LAMBART
 Antidoping inspector: (nom/name) (signature)

TO: Mr. Christian [illegible]

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY
 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: March 13, 2006

3. Date of receipt of the sample: February 27, 2006 time: 10:10 A.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 02/23/06
Race (name,stage): Amgen Tour
Place: California, USA
Discipline: Road

5. Identification of the sample examined:

 - UCI code number: **951875** Code: **7Y309**
 - external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

 - appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.022**

7. Date of analysis: February 27, 2006 - March 13, 2006

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

The following substances have been found in the sample (quantity or concentration are indicated, if applicable):

NONE

10. Enclosures: CIRCLE ONE NUMBER BELOW

 1. report of the analysis
 2. chromatogram (indicate "none" when applicable)
 3. mass spectrogram (indicate "none" when applicable)
 (4) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Michael Sekera — Certifying Scientist

Signature:

HEURE DE RECEPTION 14. MAR. 17:13 HEURE D'IMPRESSION 14. MAR. 17:16

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☑
 Hors compétition / Out of competition: ☐
2. Sexe: Masculin / Male ☑
 Sex: Féminin / Female ☐
3. Date: FEB 24 2006
4. Lieu / Place: CALIFORNIA USA
5. Course (nom, étape): AMGEN TOUR
 Race (name, stage):
6. Discipline: ROAD
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD
 First name:
 Adresse du coureur: 23356 BISHOP ROAD MURRETA CA 92562
 Address of the rider:
8. Code UCI: USA19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA CYCLING
 National federation which delivered the license:
10. Numéro de licence: 002272.
 License number:
11. Tiré au sort: Oui / Yes ☐
 Random: Non / No ☑
12. Heure de présentation: 1440
 Presentation time:
13. Heure de prélèvement: 1455.
 Time of sampling:
14. Code des flacons: 951828 A+B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris: None.
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé: Ø
Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☑
 Therapeutic use exemption (TUE) Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur:
 Assistant:
 (nom/name) (signature)
20. Médecin contrôleur: Jason Reben MD
 Examining doctor:
 (nom/name) (signature)
21. Inspecteur antidopage: William LAMBART
 Antidoping inspector:
 (nom/name) (signature)

TO: Mr. Christian [illegible]

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY
 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: March 9, 2006

3. Date of receipt of the sample: February 28, 2006 time: 10:10 A.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 02/24/06 - 02/26/06
Race (name,stage): Amgen Tour
Place: California, USA
Discipline: Road

5. Identification of the sample examined:

 - UCI code number: **951828** Code: **7Z604**
 - external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

 - appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.025**

7. Date of analysis: February 28, 2006 - March 9, 2006

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

The following substances have been found in the sample (quantity or concentration are indicated, if applicable):

NONE.

10. Enclosures: CIRCLE ONE NUMBER BELOW

 1. report of the analysis
 2. chromatogram (indicate "none" when applicable)
 3. mass spectrogram (indicate "none" when applicable)
 (4) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Yulia Kucherova
Certifying Scientist

Signature:

HEURE DE RECEPTION 10. MAR. 0:39

HEURE D'IMPRESSION 10. MAR. 0:41

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☐
 Hors compétition / Out of competition: ☐
2. Sexe: Masculin / Male ☑
 Sex: Féminin / Female ☐
3. Date: FEB. 25 2006
4. Lieu / Place: CALIFORNIA USA
5. Course (nom, étape): AMGEN TOUR
 Race (name, stage):
6. Discipline: ROAD
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD
 First name:
 Adresse du coureur: 23356 BISHOP ROAD MURRETA CA 92526
 Address of the rider:
8. Code UCI: USA19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA CYCLING
 National federation which delivered the license:
10. Numéro de licence: 002272
 License number:
11. Tiré au sort: Oui / Yes ☐
 Random: Non / No ☐
12. Heure de présentation: 1415
 Presentation time:
13. Heure de prélèvement: 1420.
 Time of sampling:
14. Code des flacons: 951866 A + B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris: NONE
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé:
Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☐
 Therapeutic use exemption (TUE) Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur:
 Assistant: (nom/name) (signature)
20. Médecin contrôleur: Richard A Johnson, M.D. Richard A Johnson, M.D.
 Examining doctor: (nom/name) (signature)
21. Inspecteur antidopage: WILLIAM LAMBART William Lambart
 Antidoping inspector: (nom/name) (signature)

USADA 0667

TO: Mr. Christian V[illegible]

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: March 9, 2006

3. Date of receipt of the sample: February 28, 2006 time: 10:10 A.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 02/24/06 - 02/26/06
Race (name,stage): Amgen Tour
Place: California, USA
Discipline: Road

5. Identification of the sample examined:

- UCI code number: **951866** Code: **7Z610**
- external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

- appearance and state of the seal: (CIRCLE A OR B)
 (a): normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.025**

7. Date of analysis: February 28, 2006 - March 9, 2006

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

The following substances have been found in the sample (quantity or concentration are indicated, if applicable):

NONE

10. Enclosures: CIRCLE ONE NUMBER BELOW

1. report of the analysis
2. chromatogram (indicate "none" when applicable)
3. mass spectrogram (indicate "none" when applicable)
(4) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Yulia Kucherova
Certifying Scientist

Signature:

HEURE DE RECEPTION 10. MAR. 0:39

HEURE D'IMPRESSION 10. MAR. 0:41

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☑
 Hors compétition / Out of competition: ☐
2. Sexe / Sex: Masculin / Male ☑ Féminin / Female ☐
3. Date: FEB 26, 2006
4. Lieu / Place: CALIFORNIA USA
5. Course (nom, étape): AMGEN TOUR
 Race (name, stage):
6. Discipline: ROAD
 Discipline:
7. Nom du coureur: LANDIS
 Surname of rider:
 Prénom: FLOYD
 First name:
 Adresse du coureur: 23356 BISHOP ROAD MURRETA CA 92562
 Address of the rider:
8. Code UCI: USA 19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA CYCLING
 National federation which delivered the license:
10. Numéro de licence: 00272
 License number:
11. Tiré au sort: Oui / Yes ☐ Non / No ☑
 Random:
12. Heure de présentation: 16 30
 Presentation time:
13. Heure de prélèvement: 1640
 Time of sampling:
14. Code des flacons: 951872 A + B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris: NONE
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé: Ø
Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☐ Non / No ☐
 Therapeutic use exemption (TUE)
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur:
 Assistant: (nom/name) (signature)
20. Médecin contrôleur: Bryan Tick
 Examining doctor: (nom/name) (signature)
21. Inspecteur antidopage: William LAMBART
 Antidoping inspector: (nom/name) William Lambart (signature)

TO: Mr. Christian [illegible]

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: March 9, 2006

3. Date of receipt of the sample: February 28, 2006 time: 10:10 A.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 02/24/06 - 02/26/06
Race (name,stage): Amgen Tour
Place: California, USA
Discipline: Road

5. Identification of the sample examined:

- UCI code number: **951872** Code: **7Z611**
- external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

- appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

Others:

6. Liquid examined: URINE

- specific gravity: **1.016**

7. Date of analysis: February 28, 2006 - March 9, 2006

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

The following substances have been found in the sample (quantity or concentration are indicated, if applicable):

NONE

10. Enclosures: CIRCLE ONE NUMBER BELOW

1. report of the analysis
2. chromatogram (indicate "none" when applicable)
3. mass spectrogram (indicate "none" when applicable)
(4.) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Yulia Kucherova — Certifying Scientist

Signature:

HEURE DE RECEPTION 10. MAR. 0:39

HEURE D'IMPRESSION 10. MAR. 0:42

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 Pinor Road, Murrieta CA 92562, U.S.A.

N° de licence :

Organisme demandant le contrôle / Organization requesting the test :
- ☒ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☐ Fédération internationale

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality :	Date de naissance / Birth Date :	Sexe / Sex :
U.S.A.	16.10.1975	M ☒ F ☐

Fédération – Discipline / Sport : CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : PARIS NICE 3ème

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date :	Heure de remise de la notification :	Lieu du contrôle / Place of test :
08 Nov 2006	16 h 10	DOUBLE TREE LIONS MARQUÉE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler :
Print name and signature of person to be tested : Floyd Landis

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VANTEVUURE MARC

Nom et prénom du médecin / Name of doctor : (en toutes lettres) KHEMISSE Alain

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival :	Date du prélèvement / Sampling date :	Heure du prélèvement / Sampling time :
16 h 40	8.11.2006	16 h 40

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 5H6013

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 125 ML

Densité / Specific Gravity : 1.020

pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal : ☐ Oui ☐ Non

Empêchement / Obstacle : ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)
PREDNISOLONE 6 cp 10-12-2006
Application locale par massage

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : OK

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :	Signature du Médecin : / Signature of Doctor :	Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 31 mars 2006

cofrac ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

27472

RAPPORT D'ANALYSE N° 55/03

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr KHENNOUF

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**Paris - Nice à St. ETIENNE**
Date :	**08/03/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**13/03/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	
Référence de(s) l'échantillon(s) :	**55/03_876013**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **14/03/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : **Recherche de substances dopantes négative**

J. de BEAURIEU
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°55/03

Résultats des rapports T/E

Référence des échantillons	T/E
876013	1.5 ✓

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP ROAD MURRIETA CA 92562

Organisme demandant le contrôle / Organization requesting the test :
- ☒ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☐ Fédération internationale

N° de licence : 0020272

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : U.S.A.

Date de naissance / Birth Date : 14.10.1975

Sexe / Sex : M ☒ F ☐

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Fédération – Discipline / Sport : CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : PARIS-NICE RASTEAU (84)

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 29/03/2006

Heure de remise de la notification : 16h20

Lieu du contrôle / Place of test : MOBILHOME - ARRIVEE RASTEAU

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested : LANDIS

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VANDENBERGHE Pierre

Nom et prénom du médecin / Name of doctor (en toutes lettres) : Dr FROIDFOND Bernard

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 16h45

Date du prélèvement / Sampling date : 29.3.2006

Heure du prélèvement / Sampling time : 16h59

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 874285

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 ML

Densité / Specific Gravity : 1.025

pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal : Oui ☐ Non ☐

Empêchement / Obstacle : Oui ☐ Non ☐

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)
PREDNICARBATE Ordonnance (07/02/06)

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : OK

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé / Signature of person tested :

Signature du Médecin / Signature of Doctor :

Signature de l'accompagnateur (éventuel) / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

LNDD
LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

REÇU le
31 MARS 2006

cofrac
ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 27 mars 2006

27437

RAPPORT D'ANALYSE N° 59/03-2

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr PROTHON

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**PARIS/NICE à RASTEAU**
Date :	**09/03/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**14/03/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**2 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**3**
Référence de(s) l'échantillon(s) :	**59/03_874285** -

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **15/03/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C*, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AUX RAPPORTS D'ANALYSE N°59/03-1, 59/03-2 et 59/03-00

Résultats des rapports T/E

Référence des échantillons	T/E
874285	1.5

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS Floyd

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 3834 Old Rd. 23356 MARIETTA (USA)

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 00208712

Type de contrôle / Type of test : OUI

Inopiné : Oui ☒ Non ☐

Nationalité / Nationality : U.S.A.

Date de naissance / Birth Date : 14.10.1975

Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : UCI Cyclisme

Nom de l'épreuve – Ville – CP / Event and place : Tour Nice Cagnes sur Mer

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 10.3.2006 — Heure de remise de la notification : 15h20 — Lieu du contrôle / Place of test : DANS le BAINS

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested : LANDIS F.

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VANDEVYVERE Axel

Nom et prénom du médecin / Name of doctor (en toutes lettres) : FRETZET Pierre

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 16h00 — Date du prélèvement / Sampling date : 10 03 2006 — Heure du prélèvement / Sampling time : 16h03

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 724264

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 120 ML

Densité / Specific Gravity : 1,010 — pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n° — Deuxième n° / Second n° — Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non — Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)
PRETINCABAT Dexamethasone (prednisolone) ou CLAVO 10C 7/3/06

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : O.K.

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé / Signature of person tested :

Signature du Médecin / Signature of Doctor :

Signature de l'accompagnateur (éventuel) / Signature of accompanying person (if any) : D. Kliauka

Exemplaire destiné au Président de la Fédération Internationale

USADA 0677

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

REÇU le
29 MARS 2006

cofrac
ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 24 mars 2006

27413

RAPPORT D'ANALYSE N° 78/03

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr FREZET

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**Paris/Nice à DIGNE LES BAINS**
Date :	**10/03/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**15/03/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**1**
Référence de(s) l'échantillon(s) :	**78/03_872364**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **16/03/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : Recherche de substances dopantes négative

J. de CEBALLOS
Directeur

Destinataires :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°78/03

Résultats des rapports T/E

Référence des échantillons	T/E
872364	1.3

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

USADA 0679

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : GANDI FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : GILMORE ROAD #335 MURRIETTA CA

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 10020772

Type de contrôle / Type of test : Inopiné : Oui ☒ Non ☐

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14.10.1975

Sexe / Sex : M ☒ F ☐

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Fédération – Discipline / Sport : CYCLISME - UCI

Nom de l'épreuve – Ville – CP / Event and place : PARIS-NICE CANNES

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 11 MARS 2006

Heure de remise de la notification : 15 H 30

Lieu du contrôle / Place of test : CANNES (FRA)

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested : Floyd Landis

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : MAYENNUCE ANEC

Nom et prénom du médecin / Name of doctor (en toutes lettres) : Dr Garcia Pluto

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 16 h 20

Date du prélèvement / Sampling date : 11.3.2006

Heure du prélèvement / Sampling time : 16 h 30

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 3715F3

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 ML

Densité / Specific Gravity : 1.020

pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)
Prednisolone (Dr Garcia Pluto) 4.37 mg

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

cofrac ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 27 mars 2006

27430

RAPPORT D'ANALYSE N° 75/03

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr CORDONNIER

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**Paris/Nice à CANNES**
Date :	**11/03/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**15/03/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**1**
Référence de(s) l'échantillon(s) :	**75/03_872357** -

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **16/03/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète - 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°75/03

Résultats des rapports T/E

Référence des échantillons	T/E
872357	1.6

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : PASEO ROAD 28356 MURRIETA (CA)

Organisme demandant le contrôle / Organization requesting the test :
- [X] Ministère chargé des Sports
- [] Direction régionale de la Jeunesse et des Sports
- [] Fédération nationale
- [] Fédération internationale

N° de licence : 00202712

Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14.10.19

Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : USCI / Cycling for fast

Nom de l'épreuve – Ville – CP / Event and place : PARIS - NICE NICE 06 000

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 12.03.06

Heure de remise de la notification : 14 h 10

Lieu du contrôle / Place of test : Bristol Hotel - Vue domicile

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler / Print name and signature of person to be tested : LANDIS F

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VAN DE VELDE Hilde

Nom et prénom du médecin / Name of doctor (en toutes lettres) : GARCIA Y Claude

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 15 h 25

Date du prélèvement / Sampling date : 12.03.06

Heure du prélèvement / Sampling time : 15 h 00

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 876044

Quantité totale d'urine, ml / Total amount of urine, ml : 125 ml

Densité / Specific Gravity : 1.020

pH / pH : 6

Échantillon insuffisant / Insufficient sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal : ☐ Oui ☐ Non

Empêchement / Obstacle : ☐ Oui ☐ Non

Médicaments / Drugs ☐ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)
Prednicarbat (Dermatop ©) pommade une fois le 7.3.06 sur le pied

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : OK

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) : Dr. Kliasdka

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE 4 0084101

USADA 0683

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

cofrac ESSAIS ACCREDITATION N° 1-1174 PORTEE COMMUNIQUEE SUR DEMANDE

Châtenay-Malabry, le 24 mars 2006

27411

RAPPORT D'ANALYSE N° 66/03-2

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr GARDIOL

Sport : Cyclisme (UCI)
Épreuve et lieu : Paris/Nice à NICE
Date : 12/03/2006

Réception de(s) l'échantillon(s) d'urine :

Date : 14/03/2006
Type de matériel : Berlinger
Nombre d'échantillon(s) : 3 (sur 4 échantillons reçus)
Nombre de rapport(s) d'analyse : 2
Référence de(s) l'échantillon(s) : 66/03_876004 -

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : 15/03/2006

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES04, ES05*) et liquide (ES03*, ES03B*) couplées ou non à la spectrométrie de masse et immunochimie (ES06).

Conclusions : Recherche de substances dopantes négative

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel.et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AUX RAPPORTS D'ANALYSE N°66/03-1 et 66/03-2

Résultats des rapports T/E

Référence des échantillons	T/E
876004	1.6

J. de CEBALLOS
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentiel et comporte 1 page(s).

USADA UNITED STATES ANTI-DOPING AGENCY
Doping Control Official Record

Floyd Landis — 4-10-06 — 6:35 AM/PM

Athlete Name Required (Place and/or Discipline if In-Competition) — Notification Date — Time

Pursuant to USADA Regulations:

- You are required to be drug tested. Signature on this form constitutes your consent to such testing.
- **Refusal to cooperate or failure to comply with the doping control process will subject you to at least a 2-year suspension and other sanctions consistent with an anti-doping rule violation.**
- You have a maximum of 60 minutes from the time of notification to arrive with your Chaperone at the Doping Control Station, unless being tested under the Out-of-Competition conditions.
- You should bring a photo ID with you to the Doping Control Station.
- Consumption of any fluid or food (even if provided by the Chaperone or a Doping Control Officer) is at your own risk.
- You may have a Representative accompany you through the testing process, but such Representative may not witness the sample collection.

KRIS FORBERG — Notifying Chaperone (Print Name)

Athlete Signature

Floyd Landis — (Print Name)

ATHLETE INFORMATION

23356 Bishop Rd. — Mailing Address

Murrieta, CA. 92562 — City, State, Zip Code, COUNTRY

951-461-8462 — Phone

4-10-06 — Arrival Date

6:35 AM/PM — Time

10-14-1975 — Date of Birth (month / day / year)

CALIFORNIA DRIVERS LIC. — Photo Identification Type

D2203288 — No.

LABORATORY/SAMPLE INFORMATION

☐ In-Competition ☒ Out-of-Competition ☐ Other

☐ Full Menu Test ☒ Partial Menu Test

Test Conducted For: n/a (or "n/a" if USADA)

4-10-06 — Sample Processing Date

8:57 AM/PM — Time

1 5 0 4 9 9 9 — SAMPLE CODE NUMBER

☒ MALE ☐ FEMALE

Specific Gravity and pH levels within range? ☒ YES ☐ NO

Specific Gravity: Greater than or equal to 1.005 (≥ 1.005)

pH: Not less than 5.0 and not greater than 7.5 ($5.0 \geq x \leq 7.5$)

OOC — Site ID Number ("OOC" if Out-of-Competition)

Cycling — Sport — Discipline (if available)

Murrieta, CA. — City/State (if OOC) — Event Name (if In-Competition)

SUBSEQUENT SAMPLE

SUBSEQUENT SAMPLE CODE NUMBER — AM/PM Time

Specific Gravity and pH levels within range? ☐ YES ☐ NO

Declaration of any recent blood transfusions, as well as any medications and other substances, including vitamins, minerals, herbs and other dietary supplements, taken during the preceding three (3) days (write "none" if none declared and draw a line through any unused spaces):

Name of Substance	Dose	Date Last Taken
Fish Oil	2 pills	4-9-06
Glucosamine	2 pills	4-9-06

Name of Substance	Dose	Date Last Taken

Consent for research (optional): By checking "I Accept" and signing in the space provided I agree that this Sample may be used for anti-doping research purposes. When all analyses have been completed, and this Sample would otherwise be discarded, it may then be used by any WADA approved laboratory for anti-doping research of any type, provided that it can no longer be identified as my Sample.

☒ I Accept ☐ I Decline

Signature:

SIGNATURES

By signing below I certify that: (i) I selected a sealed collection container and a Sample kit from the supply provided, opened and visually checked that they were empty and clean, sealed them securely and confirmed that the numbers on the Sample kit and bottles were identical to the numbers written on this *Doping Control Official Record*; (ii) the Doping Control Officer did not handle my Sample(s) except when permitted by me; (iii) no irregularities occurred in the Sample Collection Procedure; and (iv) this information is not being provided to USADA pursuant to a doctor/patient relationship and is not to be considered a confidential medical record.

Athlete Signature — Floyd Landis (Print Name)

The following individuals were present during the Sample Collection Session.

Kris Forberg — KRS FORBERG (Print Name)

Chaperone Signature

n/a — Subsequent Chaperone Signature (or "n/a" if none) — (Print Name)

Kris Forberg — KRS FORBERG (Print Name)

Doping Control Officer Signature

n/a — Language Specialist Signature (or "n/a" if none) — (Print Name)

n/a — Athlete Representative Signature (or "n/a" if none) — (Print Name)

n/a — Other Signature (or "n/a" if none)

☐ Partial Sample Form ☐ Supplementary Report Form

IOC Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave. Los Angeles, CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ACCREDITED
ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL

Attached is the report USADA7688
and the corresponding pH and specific gravity report.

regards
UCLA Olympic Analytical Laboratory

Yulia Kucherova

Tuesday, April 25, 2006

USADA7688

UCLA,USADA7688,"OOC ","These NEGATIVE urine samples were analyzed for anabolic and masking agents, diuretics, and hCG using methods 1001, 2001, and 6001."

8YZ08,A1504999,04102006,04112006,"CYCLING",T,T,F

8YZ08,A1504999,7.5,1.024

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☑
 Hors compétition / Out of competition: ☐
2. Sexe / Sex: Masculin / Male ☑ Féminin / Female ☐
3. Date: 20/4/06
4. Lieu / Place: Chattanooga, TN
5. Course (nom, étape) / Race (name, stage): Tour of Georgia
6. Discipline / Discipline: Road
7. Nom du coureur / Surname of rider: Landis
 Prénom / First name: Floyd
 Adresse du coureur / Address of the rider: 23356 Bishop Road, Murrieta, CA 92562
8. Code UCI / UCI Code: USA 19751014
9. Fédération nationale ayant délivré la licence / National federation which delivered the license: USA
10. Numéro de licence / License number: 0020272
11. Tiré au sort / Random: Oui / Yes ☐ Non / No ☑
12. Heure de présentation / Presentation time: 15:04
13. Heure de prélèvement / Time of sampling: 15:05
14. Code des flacons / Bottle codes: 951792 A + B
15. Heure de refus du coureur / Time of rider's refusal:
16. Médicaments pris / Pharmaceutical drugs taken by rider:

Contenu du livret de santé / Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) / Therapeutic use exemption (TUE) Oui / Yes ☐ Non / No ☑
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement. / Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie / Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur / Assistant: (nom/name) (signature)
20. Médecin contrôleur / Examining doctor: PETER C. RAWLINGS (nom/name) (signature)
21. Inspecteur antidopage / Antidoping inspector: Shawn Farrell (nom/name) (signature)

USADA 0690

TO: Mr. Christian Varin, UCI

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY
 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: May 10, 2006

3. Date of receipt of the sample: April 25, 2006 time: 12:00 P.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 04/18-23/06
Race (name,stage): Tour de Georgia
Place: Georgia, USA
Discipline: Road

5. Identification of the sample examined:

 - UCI code number: **951792** Code: **88R17**
 - external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

 - appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.025**

7. Date of analysis: April 25, 2006 - May 10, 2006

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

 The following substances have been found in the sample
 (quantity or concentration are indicated, if applicable):

 NONE.

10. Enclosures: CIRCLE ONE NUMBER BELOW

 1. report of the analysis
 2. chromatogram (indicate "none" when applicable)
 3. mass spectrogram (indicate "none" when applicable)
 (4). none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Yulia Kucherova
Certifying Scientist

Signature:

HEURE DE RECEPTION 11. MAI. 0:59

HEURE D'IMPRESSION 11. MAI. 1:03

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☒
 Hors compétition / Out of competition: ☐
2. Sexe / Sex: Masculin / Male ☒ Féminin / Female ☐
3. Date: 21/4/06
4. Lieu / Place: Dahlonega, GA
5. Course (nom, étape): Tour de Georgia
 Race (name, stage):
6. Discipline: Road
 Discipline:
7. Nom du coureur: Landis
 Surname of rider:
 Prénom: Floyd
 First name:
 Adresse du coureur: 23356 Bishop Rd, Murrieta, CA 92562
 Address of the rider:
8. Code UCI: USA19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA
 National federation which delivered the license:
10. Numéro de licence: 6020272
 License number:
11. Tiré au sort / Random: Oui / Yes ☐ Non / No ☒
12. Heure de présentation / Presentation time: 16:17
13. Heure de prélèvement / Time of sampling: 16:18
14. Code des flacons / Bottle codes: 951789 A+B
15. Heure de refus du coureur / Time of rider's refusal:
16. Médicaments pris / Pharmaceutical drugs taken by rider:

Contenu du livret de santé / Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) / Therapeutic use exemption (TUE): Oui / Yes ☐ Non / No ☒
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie / Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur / Assistant: (nom/name) (signature)
20. Médecin contrôleur / Examining doctor: Dr. Arun Bhavnani (nom/name) (signature)
21. Inspecteur antidopage / Antidoping inspector: Shaun Farrell (nom/name) (signature)

USADA 0692

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY
 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: May 10, 2006

3. Date of receipt of the sample: April 25, 2006 time: 12:00 P.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 04/18-23/06
Race (name,stage): Tour de Georgia
Place: Georgia, USA
Discipline: Road

5. Identification of the sample examined:

 - UCI code number: **951789** Code: **88R11**
 - external appearance of the bottle: (CIRCLE A OR B)
 (a): normal
 b: not normal, because of:

 - appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.028**

7. Date of analysis: April 25, 2006 - May 10, 2006

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

The following substances have been found in the sample (quantity or concentration are indicated, if applicable):

NONE.

10. Enclosures: CIRCLE ONE NUMBER BELOW

 1. report of the analysis
 2. chromatogram (indicate "none" when applicable)
 3. mass spectrogram (indicate "none" when applicable)
 (4) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Yulia Kucherova
Certifying Scientist

Signature:

USADA 0693

HEURE DE RECEPTION 11.MAI. 0:59 HEURE D'IMPRESSION 11.MAI. 1:03

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE

Attestation du déroulement du contrôle

ANTIDOPING CONTROL

Test certificate

1. En compétition / In competition: ☒
 Hors compétition / Out of competition: ☐
2. Sexe / Sex: Masculin / Male ☒ Féminin / Female ☐
3. Date: 22/4/06
4. Lieu / Place: Brasstown, GA
5. Course (nom, étape): Tour de Georgia
 Race (name, stage):
6. Discipline: Road
 Discipline:
7. Nom du coureur: Landis
 Surname of rider:
 Prénom: Floyd
 First name:
 Adresse du coureur: 23356 Bishop, Murrieta, CA 92562
 Address of the rider:
8. Code UCI: USA19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA
 National federation which delivered the license:
10. Numéro de licence: 0020272
 License number:
11. Tiré au sort / Random: Oui / Yes ☐ Non / No ☒
12. Heure de présentation / Presentation time: 16:40
13. Heure de prélèvement / Time of sampling: 16:48
14. Code des flacons / Bottle codes: 951787 A + B
15. Heure de refus du coureur / Time of rider's refusal:
16. Médicaments pris / Pharmaceutical drugs taken by rider:

Contenu du livret de santé / Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) / Therapeutic use exemption (TUE): Oui / Yes ☐ Non / No ☒
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie / Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur / Assistant: D. Van [illegible] (nom/name) (signature)
20. Médecin contrôleur / Examining doctor: Tom Kurisny MD (nom/name) (signature)
21. Inspecteur antidopage / Antidoping inspector: Shawn Farrell (nom/name) (signature)

USADA 0694

TO: Mr. Christian [illegible], UCI

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: May 10, 2006

3. Date of receipt of the sample: April 25, 2006 time: 12:00 P.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 04/18-23/06
Race (name,stage): Tour de Georgia
Place: Georgia, USA
Discipline: Road

5. Identification of the sample examined:

- UCI code number: **951787** Code: **88R19**
- external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

- appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.025**

7. Date of analysis: April 25, 2006 - May 10, 2006

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

The following substances have been found in the sample (quantity or concentration are indicated, if applicable):

NONE.

10. Enclosures: CIRCLE ONE NUMBER BELOW

1. report of the analysis
2. chromatogram (indicate "none" when applicable)
3. mass spectrogram (indicate "none" when applicable)
(4) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Yulia Kucherova
Certifying Scientist

Signature:

USADA 0695

HEURE DE RECEPTION 11. MAI. 0:59 HEURE D'IMPRESSION 11. MAI. 1:03

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

ANTIDOPING CONTROL
Test certificate

1. En compétition / In competition: ☒
 Hors compétition / Out of competition: ☐
2. Sexe: Masculin / Male ☒
 Sex: Féminin / Female ☐
3. Date: 23/4/06
4. Lieu / Place: Alpharetta, GA
5. Course (nom, étape): Tour de Georgia
 Race (name, stage):
6. Discipline: Road
 Discipline:
7. Nom du coureur: Landis
 Surname of rider:
 Prénom: Floyd
 First name:
 Adresse du coureur: 23356 Bishop Rd, Murrieta, CA 92562
 Address of the rider:
8. Code UCI: USA19751014
 UCI Code:
9. Fédération nationale ayant délivré la licence: USA
 National federation which delivered the license:
10. Numéro de licence: 0020272
 License number:
11. Tiré au sort: Oui / Yes ☐
 Random: Non / No ☒
12. Heure de présentation: 18:10
 Presentation time:
13. Heure de prélèvement: 18:10
 Time of sampling:
14. Code des flacons: 951790 A+B
 Bottle codes:
15. Heure de refus du coureur:
 Time of rider's refusal:
16. Médicaments pris:
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé:
Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☐
 Therapeutic use exemption (TUE) Non / No ☒
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur:
 Assistant: (nom/name) (signature)
20. Médecin contrôleur: FRED D ROSS MD
 Examining doctor: (nom/name) (signature)
21. Inspecteur antidopage: Shawn Farrell
 Antidoping inspector: (nom/name) (signature)

TO: Mr. Christian [illegible], UCI

CONFIDENTIAL

UCI ANALYSIS REPORT

1. Laboratory (name, full address): UCLA OLYMPIC ANALYTICAL LABORATORY 2122 GRANVILLE AVE. LOS ANGELES, CA, USA 90025

2. Date of this report: May 10, 2006

3. Date of receipt of the sample: April 25, 2006 time: 12:00 P.M.

4. Identification of the doping test (as on the test certificate):

Date of the race: 04/18-23/06
Race (name,stage): Tour de Georgia
Place: Georgia, USA
Discipline: Road

5. Identification of the sample examined:

- UCI code number: **951790** Code: **88R23**
- external appearance of the bottle: (CIRCLE A OR B)
 (a) normal
 b: not normal, because of:

- appearance and state of the seal: (CIRCLE A OR B)
 (a) normal
 b: not normal because of:

 Others:

6. Liquid examined: URINE

 - specific gravity: **1.025**

7. Date of analysis: April 25, 2006 - May 10, 2006

8. Methods of analysis used: GC-MS and LC-MS-MS

9. Result of analysis

The following substances have been found in the sample (quantity or concentration are indicated, if applicable):

NONE.

10. Enclosures: CIRCLE ONE NUMBER BELOW

1. report of the analysis
2. chromatogram (indicate "none" when applicable)
3. mass spectrogram (indicate "none" when applicable)
(4) none

11. Remarks (if any)

12. Name and signature of the person in charge of the analysis:

Name: Yulia Kucherova

Certifying Scientist

Signature:

USADA 0697

HEURE DE RECEPTION 11. MAI. 0:59 HEURE D'IMPRESSION 11. MAI. 1:03

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

Contrôle urinaire / Urine test ☒

1. En compétition / In competition ☒
 Hors compétition / Out of competition ☐
3. Date: 3/07/2006
5. Course (nom, étape): TOUR DE FRANCE 2ème ETAPE
 Race (name, stage):
7. Nom du coureur: LANDIS
 Surname of rider:
 Adresse du coureur: 23356 BISHOP ROAD CA 92562 MURRIETA (USA)
 Address of the rider:
8. Code UCI: USA 1975 10 14
 UCI Code:
10. Numéro de licence: 0020272
 License number:
11. Tiré au sort / Random: Oui / Yes ☒ Non / No ☐
12. Heure de présentation: 17,25
 Presentation time:
 Volume _____ pH* _____
 *Optionnel / Optional
14. Code des flacons: 995462 A + B
 Bottle codes:

ANTIDOPING CONTROL
Test certificate

Contrôle sanguin / Blood test ☐

2. Sexe: Masculin / Male ☒
 Sex: Féminin / Female ☐
4. Lieu / Place: ESCH SUR ALZETTE
6. Discipline: ROUTE G.T.
 Discipline:
 Prénom: FLOYD
 First name:
9. Fédération nationale ayant délivré la licence: USA
 National federation which delivered the license:
13. Heure de prélèvement: 17,30
 Time of sampling:
 Densité / Specific gravity* _____
15. Heure de refus du coureur: _____
 Time of rider's refusal:

16. Médicaments pris:
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé:
Contents of the health booklet:
5 05 06 Deca-Durabolin Tablets Locally 6 8 Weeks AUT
5 05 06 Methyl prednisolone Tablets Locally 6 8 Weeks AUT

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☒
 Therapeutic use exemption (TUE) Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

OK

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur: YEMIR (nom/name) (signature)
20. Médecin contrôleur: BORDABERRY GERARD (nom/name) (signature)
 Agent de prélèvement sanguin:
 Blood collection officer: (nom/name) (signature)
21. Inspecteur antidopage: MERAVIGLIA GIOVANNI (nom/name) Meraviglia Giovanni (signature)

USADA 0698

LNDD
LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

cofrac
ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 06 juillet 2006

28132

RAPPORT D'ANALYSE N° 19/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BORDABERRY

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 : 2ème étape à Esch-sur-Alzette**
Date :	**03/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**03/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**1**
Référence de(s) l'échantillon(s) :	**19/07_995462**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **04/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C*, ES04, ES05*) et liquide (ES03, ES03B*) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B*).

Conclusions : **Recherche de substances dopantes négative**

J. de CHAURAND
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°19/07

Résultats des rapports T/E

Référence des échantillons	T/E
995462	2.8

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP ROAD CA 92562 MURRIETA (USA)

N° de licence : 0020272

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14 10 1975

Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : UCI FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 9ème ETAPE

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 11/07/2006

Heure de remise de la notification : 17 10

Lieu du contrôle / Place of test : DAX (UNITE MOBILE)

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : LANDIS
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : MERAVIGLIA GIOVANNI

Nom et prénom du médecin / Name of doctor (en toutes lettres) : BORDA BERRY GERARD

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17 h 15

Date du prélèvement / Sampling date : 11/07/2006

Heure du prélèvement / Sampling time : 17 h 30

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994203

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 ml

Densité / Specific Gravity : 1,030

pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)
5 5 0 6 Decadurabolin Lundi 6 Juillet
5 5 0 6 Methylprednisolone Lundi 6 Juillet

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : Bon déletère urinaire Trois fois

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé / Signature of person tested

Signature du Médecin / Signature of Doctor :

Signature de l'accompagnateur (éventuel) / Signature of accompanying person (if any) :

Examplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE 8 0029321

USADA 0701

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

REÇU le
21 JUIL. 2006

cofrac ESSAIS
ACCRÉDITATION N° 1-1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 14 juillet 2006

28276

RAPPORT D'ANALYSE N° 99/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BORDABERRY

Sport : **Cyclisme (UCI)**
Épreuve et lieu : **TDF 2006 : 9ème étape à Dax**
Date : **11/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date : **11/07/2006**
Type de matériel : **Berlinger**
Nombre d'échantillon(s) : **3 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse : **2**
Référence de(s) l'échantillon(s) : **99/07_994203**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **12/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C*, ES04, ES05*) et liquide (ES03, ES03B*) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B*).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N° 99/07

Résultats des rapports T/E

Référence des échantillons	T/E
994203	1.3

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

ORIGINAL POUR L'U.C.I.
CONFIDENTIEL

Union Cycliste Internationale

CONTRÔLE ANTIDOPAGE
Attestation du déroulement du contrôle

Contrôle urinaire / Urine test ☒

1. En compétition / In competition ☒
 Hors compétition / Out of competition ☐
3. Date: 13/07/2006
5. Course (nom, étape): TOUR DE FRANCE 11ème ETAPE
 Race (name, stage):
7. Nom du coureur: LANDIS
 Surname of rider:
 Adresse du coureur: 23356 BISHOP ROAD CA 92562 MURRIETA (USA)
 Address of the rider:
8. Code UCI: USA 19751014
 UCI Code:
10. Numéro de licence: 0020272
 License number:
11. Tiré au sort / Random: Oui / Yes ☐ Non / No ☒
12. Heure de présentation: 18H00
 Presentation time:
 Volume 100 ml pH* 5
 *Optionnel / Optional
14. Code des flacons: 994277 A+B
 Bottle codes:

ANTIDOPING CONTROL
Test certificate

Contrôle sanguin / Blood test ☐

2. Sexe: Masculin / Male ☒
 Sex: Féminin / Female ☐
4. Lieu / Place: PLA-DE-BERET
6. Discipline: ROUTE GRAN TOUR
 Discipline:
 Prénom: FLOYD
 First name:
9. Fédération nationale ayant délivré la licence:
 National federation which delivered the license: USA
13. Heure de prélèvement: 18H20
 Time of sampling:
 Densité / Specific gravity* 1,030
15. Heure de refus du coureur:
 Time of rider's refusal:

16. Médicaments pris:
 Pharmaceutical drugs taken by rider:

Contenu du livret de santé:
Contents of the health booklet: 5 5 06 Decanethanoate Locale 6 8 week
05 05 06 Methylprednisolone Locale 6 8 Week
8 7 06 Decanethanoate Iniett 2 ml
8 7 06 Methylprednisolone 2 ml
AVT

17. Autorisation d'usage à des fins thérapeutiques (AUT) Oui / Yes ☒
 Therapeutic use exemption (TUE) Non / No ☐
18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
 Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Traces Protéines en bandelette urinaire

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy:

19. Accompagnateur: SEMIR (nom/name) (signature)
 Assistant:
20. Médecin contrôleur: BORDABERRY GERARD (nom/name) (signature)
 Examining doctor:
 Agent de prélèvement sanguin: (nom/name) (signature)
 Blood collection officer:
21. Inspecteur antidopage: MERAVIGLIA GIOVANNI (nom/name) (signature)
 Antidoping inspector:

USADA 0704

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 19 juillet 2006

28328

RAPPORT D'ANALYSE N° 121/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BORDABERRY

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 : 11ème étape à Pla-de-Beret (Espagne)**
Date :	**13/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**14/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**4 (sur 4 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**2**
Référence de(s) l'échantillon(s) :	**121/07_994277**

Résultats (Cf référentiels en vigueur : AMA 2006 : Contrôle en compétition)

Date de début des analyses : **15/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B).

Conclusions : **Recherche de substances dopantes négative**

NB : Echantillon N° 994277 : Les résultats de l'essai ES02 ne sont pas totalement exploitables en raison d'une inhibition de la dérivation due à une interférence matricielle.

J. de CEAURRIZ
Directeur

Destinataire :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N° 121/07

Résultats des rapports T/E

Référence des échantillons	T/E
994277	2.5

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS, FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : U.S.A.

Adresse / Postal adress : 23356 BISHOP ROAD, CA 92562 MURRIETA, U.S.A.

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 00202?2 / USA 1975/10/14

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : U.S.A. — Date de naissance / Birth Date : 1975/10/14 — Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : UCI FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 12ÈME ET.

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 14-07-2006 — Heure de remise de la notification : 16.30 h — Lieu du contrôle / Place of test : CARCASSONNE UNITÉ MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested : LANDIS, FLOYD

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VAN GESTEL, JAN

Nom et prénom du médecin / Name of doctor : (en toutes lettres) OLIVIER, BROCHART

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17.15 h — Date du prélèvement / Sampling date : 14-07-2006 — Heure du prélèvement / Sampling time : 17.25 h

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994276

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 cc

Densité / Specific Gravity : 1,030 — pH / pH : 5.5

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non — Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

5/5/06 Dexamethasone + Methylprednisolone AUT abroad (Local) 6-8 weeks
8/7/06 Dexamethasone + Methylprednisolone = INTRACRINOL
7/3/06 Prednisolone Persantin (Ventolin) AUT abroad 2 months

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : Signature of person tested

Signature du Médecin : Signature of Doctor :

Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE 0002922 1

USADA 0707

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

REÇU le
21 JUIL. 2006

cofrac
ESSAIS
ACCREDITATION N° 1-1174 PORTEE COMMUNIQUÉE SUR DEMANDE

Châtenay-Malabry, le 18 juillet 2006

25321

RAPPORT D'ANALYSE N° 128/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BROCHART

Sport : **Cyclisme (UCI)**
Épreuve et lieu : **TDF 2006 : 12ème étape à CARCASSONNE**
Date : **14/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date : **14/07/2006**
Type de matériel : **Berlinger**
Nombre d'échantillon(s) : **3 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse : **1**
Référence de(s) l'échantillon(s) : **128/07_994276**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **15/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C*, ES04, ES05*) et liquide (ES03, ES03B*) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B*).

Conclusions : **Recherche de substances dopantes négative**.

J. de CEAURRIZ
Directeur

Destinataires :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

** Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N°128/07

Résultats des rapports T/E

Référence des échantillons	T/E
994276	2.0

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS, FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : U.S.A.

Adresse / Postal adress : 23356 BISHOP ROAD, CA - 92562, MURRIETA, U.S.A

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 00202721 / USA 19751014

Type de contrôle / Type of test :
Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : USA

Date de naissance / Birth Date : 1975/10/14

Sexe / Sex : M ☒ F ☐

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Fédération – Discipline / Sport : UCI FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : 2ÈME TOUR DE FRANCE 15 ET

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 18-07-2006

Heure de remise de la notification : 16.10 h.

Lieu du contrôle / Place of test : L'ALPE D'HUEZ UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested : LANDIS, FLOYD

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VAN GESTEL, JAN

Nom et prénom du médecin / Name of doctor : (en toutes lettres) TOLLENAERE, BRUNO

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17.40 h.

Date du prélèvement / Sampling date : 18-07-2006

Heure du prélèvement / Sampling time : 17.50 h.

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994075

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 mL

Densité / Specific Gravity : 1,020

pH / pH : 6,0

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).

Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used : (éventuellement nom du médecin prescripteur)

5/05/2006 Dexamethasone locale Diprocel 6 - 3 semaines
5/05/2006 Methyl prednisolone locale Diprocel 6 - 3 semaines
3/7/2006 Dexamethasone intra articulaire Diprocel 2 months
3/7/2006 Methyl prednisolone intra articulaire Diprocel 2 months

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : (AUT)

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE 6 023221

USADA 0710

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 22 juillet 2006

28384

RAPPORT D'ANALYSE N° 151/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr TOLLENAERE

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 15ème étape à l'Alpe d'Huez**
Date :	**18/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**18/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**2 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**3**
Référence de(s) l'échantillon(s) :	**151/07_994075**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **19/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :

- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LNDD
LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AUX RAPPORTS D'ANALYSE N° 151/07 et 151/07-00

Résultats des rapports T/E

Référence des échantillons	T/E
994075	1.8

J. de CHAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCES-VERBAL DE CONTROLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP ROAD CA 92562 MURRIETA (USA)

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 00202782

Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14/10/1975

Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : UCI - FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 17ème ETAPE

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 20/07/2006

Heure de remise de la notification : 17 h 10

Lieu du contrôle / Place of test : MORZINE AVORIAZ UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : LANDIS
Print name and signature of person to be tested

Nom et signature du Délégué Fédéral / Name and Signature of Federal Delegate : FERRAVIGLIA Giovanni

Nom et prénom du médecin / Name of doctor : (en toutes lettres) BORDAGERAY GERARD

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17 h 50

Date du prélèvement / Sampling date : 20/07/2006

Heure du prélèvement / Sampling time : 17 h 55

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 9954714

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 125 ml

Densité / Specific Gravity : 1.030

pH / pH : 5

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :	Refus / Refusal	Oui	Non	Empêchement / Obstacle	Oui	Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

05.05.06 Doxycycline / Voie buccale / Travail
05.05.06 Ventolin / Spray buccal / Asthme
20.07.06 Xylocaine / Injection / Local
28.07.06 Prednisolone / Comprimé / Local

Commentaires sur la procédure / Comments on procedure :

Commentaires sur la procédure / Comments on procedure : Sur Bordeaux Urine Prélèvement OK

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : / Signature of person tested :

Signature du Médecin : / Signature of Doctor :

Signature de l'accompagnateur (éventuel) : / Signature of accompanying person (if any) :

0022221

VERSION NATIONALE

Exemplaire destiné au Président de la Fédération Internationale

USADA 0713

LNDD
LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

28541

Châtenay-Malabry, le 05 août 2006

cofrac
ESSAIS
ACCRÉDITATION N° 1 -1174 PORTÉE COMMUNIQUÉE SUR DEMANDE

RAPPORT DE CONTRE ANALYSE

Demandes de contre-analyse de l'UCI et de l'USADA du 31 juillet 2006 suite au rapport d'analyse n°178/07-I

Prélèvements du Dr. BORDABERRY

Organisme : **Cyclisme (UCI)**
Épreuve et lieu : **TDF 2006 : 17ème étape à MORZINE**
Date : **20/07/2006**

Réception de(s) l'échantillon(s) d'urine :
Date : **20/07/2006**
Type de matériel : **Berlinger**
Nombre d'échantillon(s) : **1 (sur 3 échantillons reçus)**
Référence de(s) l'échantillon(s) : **178/07_995474 B**

Résultats (Cf référentiels en vigueur : AMA / Réglementation nationale – contrôle en Compétition)

Date de début des analyses : **03/08/2006**

Méthodes utilisées : Chromatographie gazeuse couplée à la spectrométrie de masse GC/MS (EC24D) et Spectrométrie de masse de rapport isotopique GC/C/IRMS (EC31)

Conclusions : *(pH = 5.2 d = 1.025 +/- 0.002)*

T/E estimé à 11.0 *(variation maximale admissible = 30%)*

Concentration de Testostérone estimée après correction par la densité à 45.7 ng/mL
(Variation maximale admissible = 20 %)

Concentration d'Epitestostérone estimée après correction par la densité à 4.2 ng/mL
(Variation maximale admissible = 30 %)

Rapport supérieur au seuil de 4

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 2 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 e mail : LNDD.FRANCE@wanadoo.fr

USADA 0714

5. AUT. 2006 10:25 N° 150 P. 3

LNDD
LABORATOIRE NATIONAL DE DÉPISTAGE DU DOPAGE

RAPPORT DE CONTRE ANALYSE (SUITE)

Conclusions (Suite) :

L'analyse complémentaire par spectrométrie de masse de rapport isotopique indique une origine exogène des métabolites de la Testostérone, cohérente avec une prise de Testostérone ou de l'un de ses précurseurs.

L'origine exogène des métabolites de la Testostérone a été objectivée sur la base d'un appauvrissement isotopique de 6.4 ‰ et 3.5 ‰, respectivement pour les métabolites 5α-Androstanediol et Androstérone.

Résultat exprimé par rapport au seuil de positivité de l'AMA :

- Appauvrissement isotopique > à 3 ‰ *(variation maximale admissible appliquée au laboratoire = 0.8‰)*

J. de OLAIZOLA
Directeur

Destinataires :
- Union Cycliste Internationale – C. VARIN (CH 1860 AIGLE - SUISSE)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique– 75700 PARIS)

Pour information :
- J. SOUBLIERE : WADA (fax n°00.1.514.904.18.00)
- Dr. GENSON - Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)

* *Hors portée de l'accréditation COFRAC*

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 2 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP ROAD CA 92562 MURRIETA (USA)

N° de licence : 0028272

Nationalité / Nationality : USA

Date de naissance / Birth Date : 14/10/1975

Sexe / Sex : M ☒ F ☐

Fédération – Discipline / Sport : UCI – FFC CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 16ème ETAPE

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

Type de contrôle / Type of test :

Inopiné : Oui ☐ Non ☒

Compétition	Hors compétition
Internationale ☒	En club ☐
Nationale ☐	Stage hors club ☐
Régionale ☐	Cabinet médical ☐
	Suivi ☐

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 22/07/2006

Heure de remise de la notification : 17H30

Lieu du contrôle / Place of test : MONTCEAU LES MINES UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler / Print name and signature of person to be tested : LANDIS

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : MERAVIGLIA GIOVANNI

Nom et prénom du médecin / Name of doctor (en toutes lettres) : BORDAGBERRY GERARD

Signature du Médecin / Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 17H50

Date du prélèvement / Sampling date : 22/07/2006

Heure du prélèvement / Sampling time : 18H05

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☐

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994080

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 100 ml

Densité / Specific Gravity : 1,030

pH / pH : 6

Échantillon d'urine insuffisant / Insufficient urine sample

Scellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). / Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine) :

Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

5-5-06 Testosterone Methylpropionate 6 Dwb AUT
Cortisone Locale
8-7-06 Depo Medrol 2 ml AUT
9-7-06 Methylprednisolone 2 amp AUT
7-5-06 Prednisolone Tablets AUT

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure : Bradykinin, Urinary Tract protein

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate : Meraviglia Giovanni

Signature du sportif contrôlé / Signature of person tested :

Signature du Médecin / Signature of Doctor :

Signature de l'accompagnateur (éventuel) / Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE 8.003922 1

USADA 0716

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 26 juillet 2006

28459

RAPPORT D'ANALYSE N° 188/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr BORDABERRY

Sport : **Cyclisme (UCI)**
Épreuve et lieu : **TDF 2006 : 19ème étape à Montceau les mines**
Date : **22/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date : **22/07/2006**
Type de matériel : **Berlinger**
Nombre d'échantillon(s) : **3 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse : **2**
Référence de(s) l'échantillon(s) : **188/07_994080**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **23/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B).

Conclusions : **Recherche de substances dopantes négative**

NB : Echantillon N° 994080 : Les résultats des essais ES04 et ES05 ne sont pas totalement exploitables en raison d'une inhibition de la dérivation due à une interférence matricielle.

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N° 188/07

Résultats des rapports T/E

Référence des échantillons	T/E
994080	2.5

J. de CEAURRIZ
Directeur

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

PROCÈS-VERBAL DE CONTRÔLE ANTIDOPAGE / DOPING CONTROL FORM
CONFIDENTIEL ! / CONFIDENTIAL !

Nom et prénom du Sportif / Name of person to be tested : LANDIS, FLOYD

Nationalité de la Fédération dont relève le sportif / The Federation's nationality of the person to be tested : USA

Adresse / Postal adress : 23356 BISHOP ROAD CA 92562 MURRIETA ETATS UNIE (USA)

Organisme demandant le contrôle / Organization requesting the test :
- ☐ Ministère chargé des Sports
- ☐ Direction régionale de la Jeunesse et des Sports
- ☐ Fédération nationale
- ☒ Fédération internationale

N° de licence : 00202 72 / USA 1975 1014

Type de contrôle / Type of test : Inopiné : Oui ☐ Non ☒

Nationalité / Nationality : USA

Date de naissance / Birth Date : 1975/10/14

Sexe / Sex : M ☒ F ☐

Compétition		Hors compétition	
Internationale	☒	En club	☐
Nationale	☐	Stage hors club	☐
Régionale	☐	Cabinet médical	☐
		Suivi	☐

Fédération – Discipline / Sport : UCI - FF CYCLISME

Nom de l'épreuve – Ville – CP / Event and place : TOUR DE FRANCE 20 EME ET

Notification de contrôle et accusé de réception / Notification of testing and acknowledgement

Vous êtes convoqué(e) à un contrôle antidopage / You are hereby summoned to appear for doping test

Date / Date : 23-07-2006

Heure de remise de la notification : 17.30 H.

Lieu du contrôle / Place of test : PARIS, UNITE MOBILE

Je confirme avoir reçu et pris connaissance de cette convocation. Je sais que je serai en infraction et que je serai passible de sanctions si je ne me présente pas au contrôle, muni(e) d'une licence de compétition avec photo en cours de validité. Je prends acte que je peux me faire accompagner lors du contrôle par une personne de mon choix.

I hereby acknowledge that I have received and examined this summons. I am aware that I will be violating the charter of the organization if I do not attend the test, for which I may be sanctionned. Bring a valid identification or a valid competition card with a photo. I'm informed that I may be accompanied by one person of my choice during testing.

Nom et signature du sportif à contrôler : Print name and signature of person to be tested LANDIS, FLOYD

Nom et signature du Délégué Fédéral / Name and signature of Federal Delegate : VAN GESTEL, JAN

Nom et prénom du médecin / Name of doctor : (en toutes lettres) TOLLENAERE, BRUNO

Signature du Médecin : Signature of Doctor :

Réalisation du contrôle / Test completion

Heure d'arrivée / Time of arrival : 18.45 H

Date du prélèvement / Sampling date : 23-07-2006

Heure du prélèvement / Sampling time : 18.50 H

Identification / Identification :
- Licence / Licence : ☒
- Carte d'identité / ID : ☐
- Passeport / Passport : ☐
- Autre / Other : ☒

Échantillons A et B / Samples A and B
Code Flacon (et container) n° / bottle Code : 994171

Quantité totale d'urine, sang, ml / Total amount of urine, blood, ml : 110 mL

Densité / Specific Gravity : 1,020

pH / pH : 6.0

Échantillon d'urine insuffisant / Insufficient urine sample

Cellés intermédiaires (à utiliser en cas d'échantillon insuffisant, si moins de 75 ml d'urine). Intermediary seal number(s) (for use in the case of an insufficient sample, less than 75 ml of urine).	Premier n° / First n°	Deuxième n° / Second n°	Troisième n° / Third n°

Constat de non-venue au contrôle / The athlete failed to come for control

Signature du Médecin / Signature of Doctor :

Refus / Refusal ☐ Oui ☐ Non

Empêchement / Obstacle ☐ Oui ☐ Non

Médicaments / Drugs ☒ OUI NON ☐ (AUT)

Médicaments déclarés avoir été pris récemment / Drugs declared to have been recently used :
(éventuellement nom du médecin prescripteur)

15/05/2006 DEXAMETHASONE Locale Direct 6.3mg
15/05/2006 Methylprednisolone locale Direct 6.3mg
18/07/2006 DEXAMETHASONE infiltration Direct 2mg
19/07/2006 Methylprednisolone infiltration Direct 2mg

Commentaires sur la procédure / Comments on procedure

Commentaires sur la procédure / Comments on procedure :

Confirmation / Confirmation

Je déclare sur l'honneur que les renseignements que j'ai donnés ci-dessus sont exacts et que j'approuve la procédure de contrôle. / I declare of honour that the information I have given above is true and I approve the testing procedure.

Signature du Délégué Fédéral / Signature of Federal Delegate

Signature du sportif contrôlé : Signature of person tested :

Signature du Médecin : Signature of Doctor :

Signature de l'accompagnateur (éventuel) : Signature of accompanying person (if any) :

Exemplaire destiné au Président de la Fédération Internationale

IMPRIMERIE NATIONALE

USADA 0719

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

Châtenay-Malabry, le 26 juillet 2006

28461

RAPPORT D'ANALYSE N° 193/07

Votre demande d'analyses conventionnelles

Prélèvement(s) du Dr TOLLENAERE

Sport :	**Cyclisme (UCI)**
Épreuve et lieu :	**TDF 2006 : 20ème étape à PARIS**
Date :	**23/07/2006**

Réception de(s) l'échantillon(s) d'urine :

Date :	**23/07/2006**
Type de matériel :	**Berlinger**
Nombre d'échantillon(s) :	**3 (sur 3 échantillons reçus)**
Nombre de rapport(s) d'analyse :	**2**
Référence de(s) l'échantillon(s) :	**193/07_994171**

Résultats (Cf référentiels en vigueur : AMA/réglementation nationale : Contrôle en compétition)

Date de début des analyses : **24/07/2006**

Méthodes de dépistage utilisées : chromatographies gazeuse (ES02, ES02C, ES04, ES05) et liquide (ES03, ES03B) couplées ou non à la spectrométrie de masse, immunochimie (ES06) et colorimétrie (ES08B).

Conclusions : **Recherche de substances dopantes négative**

J. de CEAURRIZ
Directeur

Destinataires :
- Union Cycliste Internationale - C-VARIN (CH 1860 AIGLE - SUISSE)
- Dr. GENSON, Chargé d'instruction à la Fédération Française de Cyclisme (4 rue du Poète – 33700 MERIGNAC)
- Président du Conseil de Prévention et de Lutte contre le Dopage (39 rue St Dominique - 75007 PARIS)

Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de ce rapport d'analyse n'est autorisée que sous sa forme intégrale. Il est confidentiel et comporte 1 page(s).

143, avenue Roger Salengro - 92290 Châtenay-Malabry - FRANCE
Téléphone : + 33 (0)1 46 60 28 69 - Télécopie : +33 (0)1 46 60 30 17 - e-mail : direction@lndd.com

LABORATOIRE NATIONAL DE DEPISTAGE DU DOPAGE

ANNEXE AU RAPPORT D'ANALYSE N° 193/07

Résultats des rapports T/E

Référence des échantillons	T/E
994171	1.0

J. de CEAURRIZ
Directeur

. Le laboratoire n'est pas responsable du prélèvement des échantillons. La reproduction de cette annexe n'est autorisée que sous sa forme intégrale. Elle est confidentielle et comporte 1 page(s).

USADA UNITED STATES ANTI-DOPING AGENCY
Doping Control Official Record

Floyd Landis
'ete Name Required (Place and/or Discipline if In-Competition)

8-3-06
Notification Date

7:00 AM PM
Time

;uant to USADA Regulations:

- You are required to be drug tested. Signature on this form constitutes your consent to such testing.
- **Refusal to cooperate or failure to comply with the doping control process will subject you to at least a 2-year suspension and other sanctions consistent with an anti-doping rule violation.**
- You have a maximum of 60 minutes from the time of notification to arrive with your Chaperone at the Doping Control Station, unless being tested under the Out-of-Competition conditions.
- You should bring a photo ID with you to the Doping Control Station.
- Consumption of any fluid or food (even if provided by the Chaperone or a Doping Control Officer) is at your own risk.
- You may have a Representative accompany you through the testing process, but such Representative may not witness the sample collection.

Deborah Hausmaninger
Notifying Chaperone (Print Name)

Athlete Signature

Floyd Landis
(Print Name)

ATHLETE INFORMATION

23356 Bishop Rd.
Mailing Address

Murrieta, CA 92562 USA
City, State, Zip Code, COUNTRY

(951) 461-8462
Phone

8-3-06
Arrival Date

7:00 AM PM
Time

10-14-75
Date of Birth (month / day / year)

CA DL D2203288
Photo Identification Type / No.

LABORATORY/SAMPLE INFORMATION

☐ In-Competition
☒ Out-of-Competition
☐ Other

☐ Full Menu Test
☒ Partial Menu Test

Test Conducted For: N/A
(or "n/a" if USADA)

8-3-06
Sample Processing Date

8:23 AM PM
Time

1	5	0	1	8	5	0

SAMPLE CODE NUMBER

☒ MALE ☐ FEMALE

Specific Gravity and pH levels within range? ☒ YES ☐ NO
Specific Gravity: Greater than or equal to 1.005 (≥ 1.005)
pH: Not less than 5.0 and not greater than 7.5 ($5.0 \geq x \leq 7.5$)

OOC
Site ID Number ("OOC" if Out-of-Competition)

'cling
Sport / Discipline (if available)

Murrieta, CA
City/State (if OOC) / Event Name (if In-Competition)

SUBSEQUENT SAMPLE

SUBSEQUENT SAMPLE CODE NUMBER

AM PM
Time

Specific Gravity and pH levels within range? ☐ YES ☐ NO

Declaration of any recent blood transfusions, as well as any medications and other substances, including vitamins, minerals, herbs and other dietary supplements, taken during the preceding three (3) days (write "none" if none declared and draw a line through any unused spaces):

Name of Substance	Dose	Date Last Taken
Dexamethasone/Methylprednisolon	1 injec.	7-8-06
Thyroid Hormone	1 p.11	8-2-06

Name of Substance	Dose	Date Last Taken

Consent for research (optional): By checking "I Accept" and signing in the space provided I agree that this Sample may be used for anti-doping research purposes. When all analyses have been completed, and this Sample would otherwise be discarded, it may then be used by any WADA approved laboratory for anti-doping research of any type, provided that it can no longer be identified as my Sample.

☐ I Accept ☒ I Decline

Signature:

SIGNATURES

By signing below I certify that: (i) I selected a sealed collection container and a Sample kit from the supply provided, opened and visually checked that they were empty and clean, sealed them securely and confirmed that the numbers on the Sample kit and bottles were identical to the numbers written on this *Doping Control Official Record*; (ii) the Doping Control Officer did not handle my Sample(s) except when permitted by me; (iii) no irregularities occurred in the Sample Collection Procedure; and (iv) this information is not being provided to USADA pursuant to a doctor/patient relationship and is not to be considered a confidential medical record.

Athlete Signature

Floyd Landis
(Print Name)

The following individuals were present during the Sample Collection Session.

ne) Signature

VICTOR A [illegible]
(Print Name)

N/A
Language Specialist Signature (or "n/a" if none)

(Print Name)

N/A
Subsequent Chaperone Signature (or "n/a" if none)

(Print Name)

WILLIAM M GEOGHEGAN
Athlete Representative Signature (or "n/a" if none)

(Print Name)

Deborah Hausmaninger
Doping Control Officer Signature

Deborah Hausmaninger
(Print Name)

N/A
Other Signature (or "n/a" if none)

USADA 0722

☐ Partial Sample Form ☐ Supplementary Report Form

WADA Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave Los Angeles CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL
Drug Testing Report USADA7975 (Andro&Etio)

October 6, 2006

Terrence Madden
The United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906-4651
Fax 719-785-2028

USADA Site ID: OOC
Sport and Event: Cycling OOC
Collection Date: 8/3/06
Date Received at Lab: 8/4/06

Analysis: The urine sample was analyzed by the "Andro & Etio" assay using method 8001, steroids by carbon isotope ratio by GC/IRMS (see letter of June 2001 for criteria and assay details). The "Diol" assay was not attempted because the concentrations of the compounds in the samples are too low.

Analytical findings:

Labcode	Bottle#	Etiocholanolone	Androsterone	Result*
94T03	1501850	-22.1	-21.6	Negative

Unit of measurement for the two values is $\delta^{13}C$ [‰]

* Negative Result = Values NOT outside our normal ranges.

Michael Sekera
Certifying Scientist

This report shall not be reproduced, except in full, without the written approval of the laboratory.

WADA Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave Los Angeles CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL
DRUG TESTING REPORT USADA7975-EPO

Terry Madden
The United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906-4651
Fax: 719-785-2028

August 11, 2006

UCLA code: 94T
Date of laboratory receipt: August 04, 2006
Number of samples: 1
Site ID: OOC

The laboratory analyzed the specimens listed below for erythropoietic proteins (Epoetin alfa, Epoetin beta, Epoetin omega, Darbepoetin alfa) using method 9001 urinary glycoproteins by isoelectrophoresis.

UCLA code	Specimen	Result
94T03	1501850	negative

Don H. Catlin, MD
Director

This report shall not be reproduced, except in full, without the written approval of the laboratory

USADA7975

UCLA,USADA7975,"OOC ","These NEGATIVE urine samples were analyzed for anabolic and masking agents, diuretics, and hCG using methods 1001, 2001, and 6001."
94T03,A1501850,08032006,08042006,"CYCLING",T,T,F

94T03	A1501850	7	1.006

USADA UNITED STATES ANTI-DOPING AGENCY
Doping Control Official Record

Floyd Landis — 8-21-06 — 6:55 AM / PM

A Name Required (Place and/or Discipline if In-Competition) — Notification Date — Time

Pursuant to USADA Regulations:

- You are required to be drug tested. Signature on this form constitutes your consent to such testing.
- Refusal to cooperate or failure to comply with the doping control process will subject you to at least a 2-year suspension and other sanctions consistent with an anti-doping rule violation.
- You have a maximum of 60 minutes from the time of notification to arrive with your Chaperone at the Doping Control Station, unless being tested under the Out-of-Competition conditions.
- You should bring a photo ID with you to the Doping Control Station.
- Consumption of any fluid or food (even if provided by the Chaperone or a Doping Control Officer) is at your own risk.
- You may have a Representative accompany you through the testing process, but such Representative may not witness the sample collection.

KRIS FORBERG — Floyd Landis

Notifying Chaperone (Print Name) — Athlete Signature — (Print Name)

ATHLETE INFORMATION

23356 Bishop Rd. — 8-21-06 — 6:55 AM / PM

Mailing Address — Arrival Date — Time

Murrieta, CA. 92562 USA — 10-14-1975

City, State, Zip Code, COUNTRY — Date of Birth (month / day / year)

951-461-8462 — California Drivers Lic. — D3203288

Phone — Photo Identification Type — No.

LABORATORY/SAMPLE INFORMATION

☐ In-Competition ☒ Out-of-Competition ☐ Other _____

☐ Full Menu Test ☒ Partial Menu Test

Test Conducted For: n/a (or "n/a" if USADA)

8-21-06 — 7:57 AM / PM — Sample Processing Date — Time

4 9 7 1 0 4 / — ☒ MALE ☐ FEMALE

SAMPLE CODE NUMBER

Specific Gravity and pH levels within range? ☒ YES ☐ NO

Specific Gravity: Greater than or equal to 1.005 (≥ 1.005)

pH: Not less than 5.0 and not greater than 7.5 (5.0 ≥ x ≤ 7.5)

OOC

Site Number ("OOC" if Out-of-Competition)

SUBSEQUENT SAMPLE

SUBSEQUENT SAMPLE CODE NUMBER — Time AM / PM

Specific Gravity and pH levels within range? ☐ YES ☐ NO

Cycling

Sport — Discipline (if available)

Murrieta — CA.

City/State (if OOC) — Event Name (if In-Competition)

Declaration of any recent blood transfusions, as well as any medications and other substances, including vitamins, minerals, herbs and other dietary supplements, taken during the preceding three (3) days (write "none" if none declared and draw a line through any unused spaces):

Name of Substance	Dose	Date Last Taken
Thyroid Hormone	1 pill	8-20-06

Name of Substance	Dose	Date Last Taken

Consent for research (optional): By checking "I Accept" and signing in the space provided I agree that this Sample may be used for anti-doping research purposes. When all analyses have been completed, and this Sample would otherwise be discarded, it may then be used by any WADA approved laboratory for anti-doping research of any type, provided that it can no longer be identified as my Sample. ☐ I Accept ☒ I Decline

Signature: _____

SIGNATURES

By signing below I certify that: (i) I selected a sealed collection container and a Sample kit from the supply provided, opened and visually checked that they were empty and clean, sealed them securely and confirmed that the numbers on the Sample kit and bottles were identical to the numbers written on this *Doping Control Official Record*; (ii) the Doping Control Officer did not handle my Sample(s) except when permitted by me; (iii) no irregularities occurred in the Sample Collection Procedure; and (iv) this information is not being provided to USADA pursuant to a doctor/patient relationship and is not to be considered a confidential medical record.

Floyd Landis

Athlete Signature — (Print Name)

The following individuals were present during the Sample Collection Session.

Chaperone Signature — KRIS FORBERG (Print Name)

n/a — Language Specialist Signature (or "n/a" if none) — (Print Name)

n/a — Subsequent Chaperone Signature (or "n/a" if none) — (Print Name)

n/a — Athlete Representative Signature (or "n/a" if none) — (Print Name)

Doping Control Officer Signature — KRIS FORBERG (Print Name)

n/a — Other Signature (or "n/a" if none)

WADA Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave Los Angeles CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL
Drug Testing Report USADA8036 - EPO

Terry Madden
The United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906-4651
Fax: 719-785-2028

October 2, 2006

UCLA code: 9S6
Date of laboratory receipt: August 22, 2006

Number of samples: 5
Site ID: OOC

The laboratory analyzed the specimens listed below for erythropoietic proteins (Epoetin alfa, Epoetin beta, Epoetin omega, Darbepoetin alfa) using method 9001 urinary glycoproteins by isoelectrophoresis.

UCLA code	Specimen	Result
9S604	497104	negative

Michael Sekera
Negative Certifying Scientist

This report shall not be reproduced, except in full, without the written approval of the laboratory.

WADA Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave Los Angeles CA 90025
Phone (310) 825-2635 FAX (310) 206-9077

ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL
Drug Testing Report USADA8036 (9S604)

October 9, 2006

Terrence Madden
The United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906-4651
Fax 719-785-2028

Specimen number: 497104
USADA Site ID: OOC
UCLA Code: 9S604
Sport and Event: Cycling OOC
Collection Date: 8/21/06
Date Received at Lab: 8/22/06

Analysis: The urine sample was analyzed by the "Diol" assay using method 8001, steroids by carbon isotope ratio by GC/IRMS (see letter of June 2001 for criteria and assay details).

IRMS Laboratory conclusion: Values NOT outside our normal ranges.

Analytical data:	5β-adiol	5α-adiol	5β-pdiol
	-26.1	-27.9	-26.9

Unit of measurement for the three values is $\delta^{13}C$ [‰]

Michael Sekera
Certifying Scientist

This report shall not be reproduced, except in full, without the written approval of the laboratory.

USADA8036

UCLA,USADA8036,"OOC ","These NEGATIVE urine samples were analyzed for anabolic and masking agents, diuretics, and hCG using methods 1001, 2001, and 6001."
9S604,A497104,08212006,08222006,"CYCLING",T,T,F

9S604,A497104,6.0,1.023

TAB 43

IOC Working Group for Isotope Ratio Mass Spectrometry Analysis

1.Draft – confidential – 9th Nov.2001

Analytical criteria for reporting isotope ratio mass spectrometry data

Introduction

A working group of scientists from IOC accredited laboratories experienced in isotope ratio mass spectrometry (IRMS) of steroid hormones met to discuss criteria for reporting IRMS results to detect the misuse of endogenous steroid hormones.
The group considered the different reliable analytical techniques including sample preparation and instrumental conditions. The document presented set up a minimum of criteria to harmonize and report findings by IRMS techniques. Besides the criteria the group considered requirements for quality control of GC/C/IRMS.

Quality Requirements

Instrumentation

There are three companies (Micromass, PDZ Europa and Thermofinnigan) producing GC/C/IMRS instruments which are used by IOC accredited laboratories.

The reference gas (CO2) must be calibrated. For this purpose certified CO2-gas or certified reference material should be used. δ-Values ($\delta^{13}C$, ‰) will be referred to this calibrator gas.

The dynamic range (linearity) and precision of the instrument need to be established for the compounds of interest.

Reference steroids

Reference steroids should be used as internal and external standard for quality control for both intra- and inter assay. This will check the performance of the whole system (e.g. retention time, peak shape, sensitivity, combustion chamber).

Negative Quality Control Sample

The quality control sample is a negative pooled urine with historically established isotope ratios.

Batch and Injection Sequence

A batch of samples will consists of quality control sample(s) and samples being confirmed. All this samples will be processed in the same way.
The injection sequence order will consist of a solution of reference steroid(s) with low delta value(s), quality control sample(s) and the sample(s) being confirmed. If more than one sample is being confirmed then the negative quality control sample is injected before each sample set. At the end of the sequence the solution of reference steroids will be injected.

Repetition

A single sample preparation and a minimum of two injections for each sample are applied. The range in δ-values in multiple analyses (duplicate preparation or multiple injections) should not exceed 1,5 ‰.

Decision Criteria

Each laboratory using GC/C/IRMS should determine its own population reference range with a minimum of 50 volunteer samples from healthy subject without using therapeutic and by IOC banned substances.
Analytes for indicating the misuse of endogenous substances (e.g. androsterone, etiocholanolone, 5α- and 5ß-diols, etc.) as well as endogenous reference compounds (ERC) not affected by the misuse of endogenous steroid hormones (e.g. pregnanediol, pregnanetriol, 11-ketoetiocholanolone, etc.) should be measured.

From the measured δ-values (corrected or not corrected for derivatisation) the following reference ranges must be established: The δ-values, the difference of δ-values and the ratio between analyte and ERC.
Correction for derivatisation is necessary when analyte and ERC have different number of introduced carbon atoms (e.g. acetates of androsterone and pregnanediol). Correction for derivatisation is preferably performed for all substances following a mass balance equation as described by Docherty et al. in Rapid Commun Mass Spectrom 2001, 15: 730-738.
This correction will facilitate the data exchange between laboratories.

The population reference range will be calculated with 99.9% confidence interval (e.g. mean ± 3SD for normal distrubution). Reference: Recommendation of the International Federation Clinical Chemistry (IFCC), Solberg et al.: Reference values. Advances in Clin Chem 27, 1989: 1-79.

A sample will be considered as analytical positive when the difference or the ratio of δ-values between analyte and ERC is outside the population reference range.
If only the absolute δ-value(s) of the analyte(s) is outside the population reference range additional investigations should be undertaken.

Proposal for reporting IRMS results

For a positive result the working group propose the following wording (e.g. in case of androsterone, eticholanolone as analytes and pregnanediol as ERC):

"The decreased isotope values ($\delta^{13}C$) of androsterone and etiocholanolone in comparison to that of the endogenous reference compound pregnanediol indicate the use of a banned endogenous anabolic androgenic steroid such as testosterone, 4-androstenedione, 5-androstenediol, DHEA, etc."

If in subsequent reports isotope data are included they should be quoted in terms of the δ-notation and refer to the PDB standard (Craig, Geochem Cosmochim Acta 1957, 12:133; further informations are available at http://www.iaea.org/worldatom/publications/catalogue.html).

① Difference should cancel deriv. contribution if # of C's the same.

Analytical Criteria for IRMS

IRMS - Isotope Ratio Mass Spectrometry

- IOC working group

Willi Schänzer
Ray Kaslauskas
Christiane Ayotte
Makoto Ueki
Don Catlin
Xavier de la Torre

- 1st Meeting in Cologne 2./3.October 2001

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

The working group considered

- Different reliable analytical techniques including sample preparation and instrumental conditions
- Set up a minimum of criteria to harmonize and report findings by IRMS techniques
- Besides the criteria the group considered requirements for quality control of GC/C/IRMS

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

- Quality Requirements
- Decision Criteria
- Reporting

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Quality Requirements

- *Instrumentation*
- *Reference gas*
- *Linearity, Precision*
- *Reference Steroids*
- *Negative Quality Control Sample*
- *Batch and Injection Sequence*
- *Repetition*

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Quality Requirements

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality Control Sample
Batch and Injection Sequence
Repetition

- ***Instrumentation***
 Micromass, PDZ Europa and Thermofinnigan
- ***Reference gas***
 The reference gas (CO_2) must be calibrated.
 For this purpose certified CO_2-gas or certified reference material should be used.
 δ-Values ($\delta^{13}C$, ‰) will be referred to this calibrator gas.

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Quality Requirements

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality Control Sample
Batch and Injection Sequence
Repetition

- ***Instrumentation***
 Micromass, PDZ Europa and Thermofinnigan
- ***Linearity, Precision***
 The dynamic range (linearity) and precision of the instrument need to be established for the compounds of interest..

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Quality Requirements

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality Control Sample
Batch and Injection Sequence
Repetition

- ***Reference Steroids***
 Reference steroids should be used as internal and external standard for quality control for both intra- and inter assay.
 This will check the performance of the whole system (e.g. retention time, peak shape, sensitivity, combustion chamber).

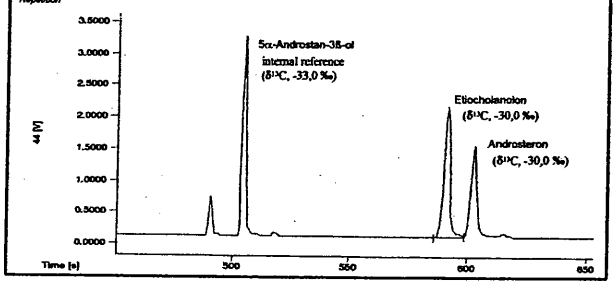
Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Quality Requirements ▪ ***Reference Steroids***

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality Control Sample
Batch and Injection Sequence
Repetition



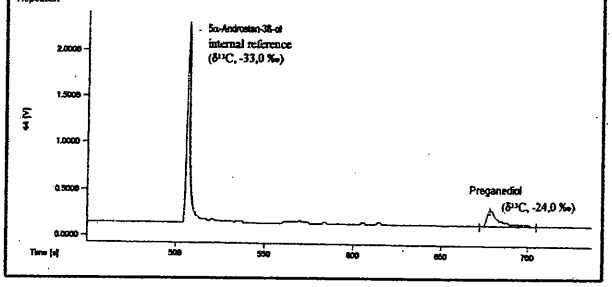
Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality
Control Sample
Batch and
Injection Sequence
Repetition

IRMS - isotope ratio mass spectrometry

Quality Requirements ▪ ***Reference Steroids***



Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality
Control Sample
Batch and
Injection Sequence
Repetition

IRMS - isotope ratio mass spectrometry

Quality Requirements

▪ ***Negative Quality Control Sample***

The quality control sample is a negative pooled urine with historically established isotope ratios.

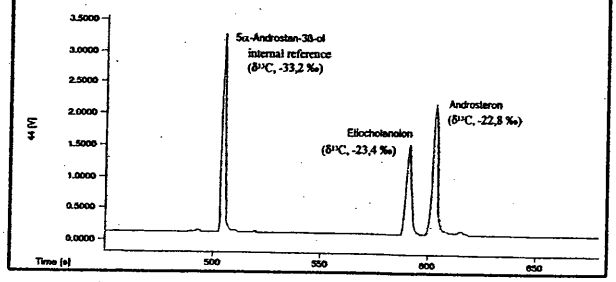
Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality
Control Sample
Batch and
Injection Sequence
Repetition

IRMS - isotope ratio mass spectrometry

Quality Requirements ▪ ***Negative Quality Control Sample***



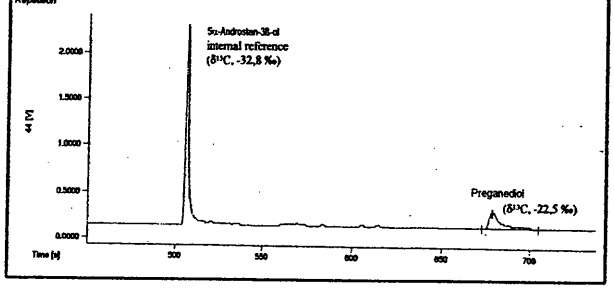
Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality
Control Sample
Batch and
Injection Sequence
Repetition

IRMS - isotope ratio mass spectrometry

Quality Requirements ▪ ***Negative Quality Control Sample***



Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Quality Requirements

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality Control Sample
Batch and Injection Sequence
Repetition

- ***Batch and Injection Sequence***

A **batch** of samples will consists of
- quality control sample(s) and
- samples being confirmed.

All this samples will be processed in the same way.

The **injection sequence** order will consist of a
- solution of reference steroid(s) with low delta value(s),
- quality control sample(s) and
- the sample(s) being confirmed.

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

1. Reference steroids w	2. Quality control sample	3. Sample to be confirmed	4. Reference steroids w

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality Control Sample
Batch and Injection Sequence
Repetition

- The **injection sequence** order will consist of a
 - solution of reference steroid(s) with low delta value(s),
 - quality control sample(s) and
 - the sample(s) being confirmed.

If more than one sample is being confirmed then the negative quality control sample is injected before each sample set.

At the end of the sequence the solution of reference steroids will be injected.

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

1. Reference steroids w	2. Quality control sample	3. Sample to be confirmed	4. Quality control sample	5. Sample to be confirmed	6. Reference steroids w

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality Control Sample
Batch and Injection Sequence
Repetition

- The **injection sequence** order will consist of a
 - solution of reference steroid(s) with low delta value(s),
 - quality control sample(s) and
 - the sample(s) being confirmed.

If more than one sample is being confirmed then the negative quality control sample is injected before each sample set.

At the end of the sequence the solution of reference steroids will be injected.

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Quality Requirements

Instrumentation
Reference gas
Linearity, Precision
Reference Steroids
Negative Quality Control Sample
Batch and Injection Sequence
Repetition

- ***Repetition***

A single sample preparation and a minimum of two injections for each sample are applied.

The range in δ-values in multiple analyses (duplicate preparation or multiple injections) should not exceed 1,5 ‰. .

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Decision Criteria

- *Reference Population*
- *Analytes*
- δ-values, etc.
- *Correction of* δ-values
- *Calculation of* Reference Population
- *Analytical Positive*

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Decision Criteria

Reference Population
Analytes
δ-values, etc.
Correction of δ-values
Calculation of Reference Population
Analytical Positive

- *Reference Population*

Each laboratory using GC/C/IRMS should determine its own population reference range with a minimum of 50 volunteer samples from healthy subject without using therapeutic and by IOC banned substances.

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Decision Criteria

Reference Population
Analytes
δ-values, etc.
Correction of δ-values
Calculation of Reference Population
Analytical Positive

- *Analytes*

Analytes for indicating the misuse of endogenous substances (e.g. androsterone, etiocholanolone, 5α- and 5β-diols, etc.)
as well as *endogenous reference compounds (ERC)* not affected by the misuse of endogenous steroid hormones
(e.g. **pregnanediol, pregnanetriol, 11-ketoetiocholanolone**, etc.) should be measured..

Manfred Donike
Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Decision Criteria

Reference Population
Analytes
δ-values, etc.
Correction of δ-values
Calculation of Reference Population
Analytical Positive

- δ-values, etc.

From the measured δ-values (corrected or not corrected for derivatisation)
the following reference ranges must be established:
- the δ-values,
- the difference of δ-values and
- the ratio between analyte and ERC.

Manfred Donike Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Decision Criteria

Reference Population
Analytes
δ-values, etc.
Correction of δ-values
Calculation of Reference Population
Analytical Positive

- ***Correction of* δ-values**

Correction for derivatisation is necessary when analyte and ERC have different number of introduced carbon atoms
(e.g. acetates of **androsterone** and pregnanediol).
Correction for derivatisation is preferably performed for all substances following a mass balance equation as described by *Docherty et al. in Rapid Commun Mass Spectrom 2001, 15: 730-738.*

This correction will facilitate the data exchange between laboratories.

Manfred Donike Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Decision Criteria

Reference Population
Analytes
δ-values, etc.
Correction of δ-values
Calculation of Reference Population
Analytical Positive

- **Reference Population**

The population reference range will be calculated with 99.9% confidence interval
(e.g. mean ± 3SD for normal distrubution)

Reference:
Recommendation of the International Federation Clinical Chemistry (IFCC), Solberg et al.: Reference values. Advances in Clin Chem 27, 1989: 1-79.

Manfred Donike Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Decision Criteria

Reference Population
Analytes
δ-values, etc.
Correction of δ-values
Calculation of Reference Population
Analytical Positive

- ***Reference Population***

Example: Aguilera et al., Clin Chem 47 (2001) 292-300

Table 3. Control group of 73 subjects: Descriptive statistics for $\delta^{13}C$, differences between the means, and ratios of the means.

	$\delta^{13}C$, ‰			Difference, ‰		Ratio	
	SpA	SwA*	SdP*	SdP − SpA	SpP − SwA	SpA/SdP	SwA/SdP
Mean	−25.69	−26.35	−24.26	1.43	2.09	1.06	1.09
SD	0.92	0.68	0.70	0.68	0.63	0.028	0.027
CV, %	3.6	2.6	2.9			2.7	2.5
Mean + 3 SD	−22.92	−24.31	−22.15	3.47	3.99	1.14	1.17
Mean − 3 SD	−28.46	−28.39	−26.37	−0.62	0.18	0.97	1.00
Maximum	−23.50	−24.55	−22.92	3.17	3.72	1.13	1.16
Minimum	−27.82	−27.89	−25.49	−0.08	0.15	1.00	1.01
Max − Min	3.9	3.3	2.6				

* Mean significantly different from SpA.
* Mean significantly different from SpA and SwA.

Manfred Donike Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Decision Criteria

Reference Population
Analytes
δ-values, etc.
Correction of δ-values
Calculation of Reference Population
Analytical Positive

- ***Analytical Positive***

A sample will be considered as analytical positive when the difference or the ratio of δ-values between analyte and ERC is outside the population reference range.

e.g.
Androsterone −32,0 ‰
Pregnanediol −30,1 ‰

If only the absolute δ-value(s) of the analyte(s) is outside the population reference range additional investigations should be undertaken.

Manfred Donike Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Reporting

- *Proposal for reporting IRMS results*

For a positive result the working group propose the following wording (e.g. in case of androsterone, eticholanolone as analytes and pregnanediol as ERC):

"The decreased isotope values ($\delta^{13}C$) of androsterone and etiocholanolone in comparison to that of the endogenous reference compound pregnanediol indicate the use of a banned endogenous anabolic androgenic steroid such as testosterone, 4-androstenedione, 5-androstenediol, DHEA, etc."

Manfred Donike Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Reporting

- *Proposal for reporting IRMS results*

If in subsequent reports isotope data are included they should be quoted in terms of the δ-notation and refer to the Pee Dee belemnite standard (PDB)

Craig,
Geochem Cosmochim
Acta 1957, 12:133;

further informations are available at
http://www.iaea.org/worldatom/publications/catalogue.html

Manfred Donike Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

Next steps

- *Estimation of reference Population in each lab using GC/C/IRMS*
- Discussion of the results by end of September or later (?),
 possible modifications of the criteria
 preparation of final version of the document
- **Publication by the IOC**

Manfred Donike Workshop 2002

Analytical Criteria for IRMS

IRMS - isotope ratio mass spectrometry

- **IOC working group**

Willi Schänzer
Ray Kaslauskas
Christiane Ayotte
Makoto Ueki
Don Catlin
Xavier de la Torre

- 2nd Meeting in ?

ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 645–650

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

$^{13}C/^{12}C$ Isotope ratio MS analysis of testosterone, in chemicals and pharmaceutical preparations

X. de la Torre [a,c], J.C. González [a], S. Pichini [b], J.A. Pascual [a,d], J. Segura [a,d,*]

[a] *Pharmacology Research Unit, Institut Municipal d'Investigaciò Medica, Doctor Aiguader 80, E-08003 Barcelona, Spain*
[b] *Istituto Superiore di Sanitá, Roma, Italy*
[c] *Universitat Autónoma de Barcelona, Spain*
[d] *Universitat Pompeu, Fabra, Barcelona, Spain*

Received 9 February 2000; received in revised form 31 May 2000; accepted 10 September 2000

Abstract

The $^{13}C/^{12}C$ ratio can be used to detect testosterone misuse in sport because (semi)-synthetic testosterone is supposed to have a ^{13}C abundance different from that of endogenous natural human testosterone. In this study, gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) analysis for the measurement of the δ ^{13}C ‰ value of testosterone from esterified forms of 13 pharmaceutical preparations, six reagent grade chemicals and three bulk materials (raw materials used in pharmaceutical proarations) obtained world-wide was investigated after applying a strong acidic solvolytic procedure. Mean δ ^{13}C‰ values of non esterified (free) testosterone from chemicals and bulk materials of several testosterone esters were in the range: $-25.91/-32.82$‰ while the value obtained for a (semi)-synthetic, reagent grade, free testosterone was -27.36‰. The δ ^{13}C‰ results obtained for testosterone from the pharmaceuticals investigated containing testosterone esters were quite homogeneous (mean and S.D. of δ ^{13}C‰ values of free testosterone: 27.43 ± 0.76‰), being the range between -26.18 and -30.04‰. Values described above were clearly different from those reported by several authors for endogenous natural human testosterone and its main metabolites excreted into the urine in non-consumers of testosterone (δ ^{13}C‰ range: from -21.3 to -24.4‰), while they were similar to those of urinary testosterone and metabolites from individuals treated with testosterone esters and testosterone precursors. This finding justifies the fact that administration of these pharmaceutical formulations led to a statistical decrease of carbon isotope ratio of urinary testosterone and its main metabolites in treated subjects.
© 2001 Elsevier Science B.V. All rights reserved.

Keywords: Synthetic testosterone; GC/C/IRMS analysis; δ ^{13}C‰ value; Pharmaceutical preparations; Sports drug testing

1. Introduction

Recent studies indicate that (semi)-synthetic testosterone seems to present a different $^{13}C/^{12}C$ ratio, when compared to human endogenous compound [1]. This observation is based on the fact

* Corresponding author. Tel.: +34-93-2211009; fax: +34-93-2213237.
E-mail address: jsegura@imim.es (J. Segura).

0731-7085/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(00)00452-0

that endogenous testosterone reflects an average of all the carbon from vegetal and animal material eaten by humans, while synthetic testosterone is usually elaborated from a single plant species starting material (phytosterols) with a defined value of $^{13}C/^{12}C$ ratio fixed from its own mechanism of discrimination from atmospheric CO_2. The plant species commonly used is soy, which exhibits lower ^{13}C content as compared with human produced testosterone [2].

Differences in $^{13}C/^{12}C$ ratio can be determined by carbon isotope ratio mass spectrometry (IRMS) [3,4]. For complex mixtures, this technique can be coupled with a gas chromatograph via a combustion interface (GC/C). Separated organic analytes are combusted to CO_2 and the analysis of the $^{13}CO_2$ and $^{12}CO_2$ molecules allows the calculation of carbon isotope ratios for each compound [5]. Variations of the heavier isotope are of the order of 0.001–0.05 atoms % [4]. These minute differences in carbon isotope ratios are expressed as a per mil deviation compared to a designated isotopic standard using the δ $^{13}C‰$ notation:

$$^{13}C‰ = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where R_{sample} is the measured $^{13}C/^{12}C$ isotope ratio for the sample and $R_{standard}$ is the measured $^{13}C/^{12}C$ isotope ratio for a defined standard. The international standard for C is a sample of $CaCO_3$, obtained from the Pee Dee formation in South Carolina (Pee Dee belemnite, PDB) with an accepted value of $R_{standard} = 0.0112372 \pm 0.000009$ [2]. In practice, calibrated CO_2 gas or substances (e.g. alcanes) with a certified δ values are used as a reference standard.

In recent years, the use of GC/C/IRMS has been investigated for the detection of the misuse of testosterone and other endogenous steroids in sport [6,7]. Indeed, different studies showed that following the administration of testosterone and related compounds (androstenedione, dehydroepiandrosterone or androstendiols), carbon isotope ratio of urinary testosterone and its main metabolites declined significantly with differences of more than 3‰ units on the δ-scale [8–10]. These results assumed that synthetic sources of testosterone presented a lower ^{13}C content than naturally occurring steroids.

Synthetic testosterone is usually present in pharmaceutical preparations as esterified forms, which are administered orally or intramuscularly for primary male hypogonadism or breast cancer therapy and can be obtained by medical prescription.

To date, only δ $^{13}C‰$ values of a limited number of pharmaceutical preparations of synthetic testosterone esters have been reported but no detailed information was given about the products (type of esterified compound, name of the formulation, etc), or whether the measurements were carried out directly on the esters or using the eventual hydrolysis of esterified forms to obtain free testosterone, which is the substance of interest to be analysed by GC/C/IRMS [11,12].

In this work, the δ $^{13}C‰$ value of free testosterone obtained after cleavage of different esterified forms from 13 pharmaceutical preparations, six reagent grade chemicals and three bulk materials (raw materials used in pharmaceutical preparations) obtained worldwide was investigated using GC/C/IRMS analysis.

2. Experimental

2.1. Materials

Testosterone esters reagent grade chemicals were obtained from Sigma (St Louis, MO) and Research Plus (Bayonne, NJ). Testosterone esters bulk materials were from Organon Española S.A. (Sant Boi de Llobregat, Barcelona, Spain). Testosterone esters pharmaceutical preparations were obtained from China, Japan, Germany, Greece, Sweden, Portugal, Italy, Norway and Czech Republic by local authorised dealers (see Table 2 for specific information). Standards of *n*-octadecane and *n*-tricosane with a certified δ $^{13}C‰$ value were purchased from Chiron (Trondheim, Norway). Testosterone and 5α-androstane-3β-ol reagent grade chemicals were purchased from Sigma (St Louis, MO). All other reagents were of analytical grade.

USADA 0743

2.2. Treatment of testosterone esters in chemicals, bulk materials and pharmaceutical preparations for GC/C/IRMS analysis of non esterified (free) testosterone

Reagent grade chemicals and bulk materials were directly subiected to strong acidic solvolysis. A 400 μl volume of testosterone esters methanolic solution (10 μg/ml) were mixed with 1 ml ethyl acetate–methanol–concentrated sulphuric acid 100:20:5 v/v/v and incubated at 55°C overnight under nitrogen atmosphere. After incubation, samples were added with two drops of 20% ammonia agueous solution, 1 ml acetate buffer (final pH 5.2) and 2 μg 5α-androstane-3β-ol as internal standard and then extracted with three aliquots of 2 ml *n*-hexane. In the case of pharmaceutical formulations, which contained testosterone esters mixed with oily excipients (e.g. ricin oil, arachid oil, oleic acid, benzyl benzoate), an appropriate volume was sampled and diluted with *n*-hexane in order to have stock solutions of 100 μg/ml esters. A 40 μl volume of this solution underwent strong acidic solvolysis as described above. After solvolysis, samples were treated and extracted as reported above. Final extracts (from chemicals, bulk materials or pharmaceutical preparations) were evaporated under nitrogen stream at 45°C, dried out under vacuum (600 mbar) over phosphorous pentoxide for at least 20 min and redissolved in 70 μl ciclohexane. Two microlitres of this non-derivatised solution were directly injected into the gas chromatograph/combustion/carbon isotope ratio mass spectrometer system (GC/C/IRMS).

2.3. GC/C/IRMS analysis

Carbon isotope ratio measurements were performed using a Finnigan MAT Delta Plus instrument (Finnigan MAT GmbH, Bremen, Germany). The GC (Hewlett–Packard HP 6890) was equipped with an HP1 column (17 m, 0.2 mm i.d., 0. 11 μm film thickness, Hewlett–Packard) with helium as the carrier gas and injections were made in splitless mode using an open liner with a 5 mm plug of silanised glass wool inside. Constant flow through the column was set at 2 ml/min. The GC oven was programmed from 150 (1 min) to 260°C (30°C/min), then to 290°C (1.5°C/min) and finally increased to 300°C (5°C/min). A glass three-way connector (BGB analytik AG, Anwil, Switzerland) was used to connect the GC column to the fused silica capillaries leading to the combustion oven and backflush valve. Oxidation and reduction reactors operated respectively at 940 and 600°C.

During routine operation, determination of δ ^{13}C‰ values was done using pulses of CO_2 gas (δ ^{13}C‰ -37.50), previously calibrated against *n*-octadecane and *n*-tricosane certified standards (δ ^{13}C‰ of -30.71 and -26.71, respectively). CO_2 gas was pulsed directly into the ion source during the backflush period (solvent elution) for five times. The first two were used to stabilise the system, the third one was taken for the calibration and the last two to back-check for the calibration values.

2.4. Statistics

Experimental data were statistically analysed with common statistical packages using one factor ANOVA. Results were considered statistically different when $P < 0.05$.

3. Results and discussion

Testosterone is usually administered as a prodrug (e.g. as 17-β-hydroxy-esters). Nonetheless, for the purpose of sports drug testing, a study on δ ^{13}C‰ values of testosterone in esterified form, as reported by some authors [12], appeared to be of minor relevance since, physiologically, free testosterone is the active compound and the usual target analyte in extracts of human urine. The knowledge of δ ^{13}C‰ value of non-esterified (free) testosterone obtained from testosterone esters of synthetic origin was considered essential as only free compound participated in the catabolic pathway of endogenous steroids.

USADA 0744

3.1. Solvolysis procedure

Different procedures to obtain free testosterone were evaluated (data not shown) on testosterone esters reagent grade chemicals: an alkaline hydrolysis (20% KOH in absolute ethanol), an enzymatic hydrolysis with β-glucuronidase enzyme, a mild acidic solvolysis (ethyl acetate–methanol–concentrated sulphuric acid 100:20:1 v/v/v) and a strong acidic solvolysis (ethyl acetate–methanol–concentrated sulphuric acid 100:20:5 v/v/v). A GC/MS analysis with a standardised methodology [13] showed that consistently higher hydrolysis yields (> 97%) were obtained with strong acidic solvolysis. Thus, that procedure was chosen, for the treatment of testosterone esters chemicals, bulk materials and pharmaceutical preparations before GC/C/IRMS analysis.

3.2. GC/C/IRMS analysis

3.2.1. δ ^{13}C‰ values of free testosterone obtained after solvolysis of different testosterone esters (chemicals and bulk materials)

Table 1 reports δ ^{13}C‰ values of free testosterone obtained after strong acidic solvolysis of several testosterone esters (chemicals and bulk materials) as well as the value obtained for testosterone (reagent grade chemical) concurrently injected. Values obtained (range from −25.91 to −32.82‰; mean ± S.D. −28.81 ± 2.10‰), were always lower (more negative) than −25‰ in agreement with values reported by other authors for commercially available testosterone [7,11]. From the manufacturers checked, only Research Plus showed a statistically significant difference from the others. Probably such differences can be explained by the different phytosteroids coming from plants of different species or geograhical areas used as a starting material for synthesis. Even though these differences could account for such inter-supplier statistical variations, differences among various testosterone esters were also found for each manufacturer.

3.2.2. δ ^{13}C‰ values of free testosterone obtained after solvolysis of testosterone esters from pharmaceutical preparations

Table 2 shows the δ ^{13}C‰ values of free testosterone obtained from testosterone esters in pharmaceutical preparations. A narrower range of values (from −26.80 to −28.45 ‰ with mean ± S.D. −27.43 ± 0.76‰) was found as compared with chemicals and bulk materials of testosterone esters. Nevertheless, in case of testosterone enanthate, statistically significant differences were found between batches of the same product (i.e. Testoviron Depot from Schering, Germany) and among the different manufacturers. Conversely, for testosterone undecanoate, batches from the

Table 1
δ ^{13}C‰ value of free testosterone obtained after solvolysis of testosterone esters as chemicals and bulk materials

Substance	Batch number	Supplier	δ ^{13}C‰ value (free testosterone)[a]
Reagent grade chemicals			
Testosterone cypionate	128FO620	Sigma	−26.77 ± 0.41
Testosterone enanthate	35FO663	Sigma	−28.43 ± 1.40
Testosterone propionate	10H0308	Sigma	−28.67 ± 1.11
Testosterone hemisuccinate	7494	Research Plus	−29.12 ± 0.58
Testosterone undecanoate	14389	Research Plus	−32.82 ± 1.03
Testosterone valerate	3-125	Research Plus	−31.07 ± 0.44
Pure free testosterone	126F-0426	Sigma	−27.36 ± 0.60
Bulk materials			
Testosterone decanoate	06586	Organon	−25.91 ± 0.47
Testosterone isocaproate	06626	Organon	−27.68 ± 0.76
Testosterone phenylpropionate	06587	Organon	−28.81 ± 1.14

[a] Mean ± S.D. of six replicates

USADA 0745

Table 2
δ $^{13}C‰$ value of free testosterone obtained after solvolysis of testosterone esters from pharmaceutical preparations

Active principle	Manufacturer	Authorised dealer	Commercial name	Batch number	Pharmaceutical form	δ $^{13}C‰$ value (mean ± S.D.)
Testosterone enanthate	Schering (Germany)	Schepa (Greece)	Testoviron Depot	72496	Oily i.m. injectable	−26.80 ± 0.23
	Schering (Germany)	Schering Lusitana (Portugal)	Testoviron Depot	73502	Oily i.m. injectable	−28.45 ± 0.78
	Fuji Pharmaceuticals (Japan)	Fuji Pharmaceuticals (Japan)	Testron Depot-S	N.A.[a]	Oily i.m. injectable	−27.21 ± 0.34
	Jenapharm (Germany)	Jenapharm (Germany)	Testosteron Depot	73807	Oily i.m. injectable	−26.82 ± 0.41
	Rotexmedica (Germany)	Rotexmedica (Germany)	Testosteron Depot	70471	Oily i.m. injectable	−27.40 ± 0.28
Testosterone propionate	Spofa (Czech Republic)	Spofa (Czech Republic)	Agovirin	3020294	Oily i.m. injectable	−27.38 ± 0.55
	9th Pharmaceutical Factory (China)	9th Pharmaceutical Factory (China)	Tongyong	970104	Oily i.m. injectable	−27.00 ± 0.16
Testosterone undecanoate	N-V.Organon Oss (Holland)	Organon (Greece)	Restandol	070421	Oily capsules	−27.40 ± 0.45
	N.V.Organon Oss (Holland)	Organon (Portugal)	Andriol	113443	Oily capsules	−28.32 ± 1.05
	N.V.Organon Oss (Holland)	Organon A/S (Norway)	Androxon	A17511	Oily capsules	−27.46 ± 1.09
	N.V.Organon Oss (Holland)	Oy Organon Ab (Sweden)	Panteston	A20296	Oily capsules	−27.15 ± 0.46
	Organon (Germany)	Organon (Germany)	Andriol	4416101	Oily capsules	−27.50 ± 0.70
	Scherer (Italy)	Organon (Italy)	Andriol	957904	Oily capsules	−27.78 ± 0.80

[a] N.A., data not available.

USADA 0746

same manufacturer (N.V.Organon Oss, Holland) did not present statistical differences and no other factors such as authorised dealer or country were found to significantly contribute to differences in δ ^{13}C‰ value. For testosterone propionate, no difference was obtained between the two products analysed. In any case, values of δ ^{13}C‰ obtained were in agreement with those reported by Shackleton and co-workers [10], once corrected for the contribution of the acetyl group introduced in the molecule as derivative by those authors. Interestingly, values presented in Tables 1 and 2 ('synthetic' testosterone) were different from those found for endogenous testosterone and its main metabolites extracted from urine in non consumers of testosterone (δ ^{13}C‰ range: $-21.3/-24.4$‰) as reported by several authors [7,11,14]. Conversely, δ ^{13}C‰ values of free testosterone obtained after solvolysis of testosterone esters as chemicals, bulk materials and from pharmaceutical preparations were similar to those of urinary testosterone and metabolites of individuals treated with testosterone esters and testosterone precursors [6–15].

In conclusion, carbon isotope mass spectrometry appeared to be an effective mean to distinguish synthetic testosterone coming from reagent grade chemicals, bulk materials or pharmaceutical preparations from human endogenous compound or its urinary metabolites. This finding justify the fact that administration of these pharmaceutical formulations led to a statistical decrease of carbon isotope ratio of urinary testosterone and its main metabolites in treated subjects.

Acknowledgements

The authors acknowledge Dr C. Georgakopolulos, Greece; Dr P. Hemmersbach, Norway; Dr K. Kuoppasalmi, Finland; Dr L. Lourenço Reys, Portugal; Dr K. Muller, Germany; Dr R. Nicoletti, Italy; Dr R. Slechtová, Czech Republic; Dr M. Ueki, Japan; Dr M. Wu, P.R. of China, from International Olympic Committee accredited laboratories, which provided reference materials. Support from International Olympic Committee Medical Commission (Prince A. de Merode, Dr P. Schamasch), Ministerio de Educación y Ciencia, Spain (project PB98-1628) and European Commission (project G6RD CT99 00149) is acknowledged.

References

[1] G. Southan, A. Mallat, J. Jumeau, S. Craig, N. Poojara, D. Mitchell, et al., Programme and Abstracts of the Second International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona, 1990, 1990, p. 306.
[2] B.N. Smith, S. Epstein, Plant Physiology 47 (1971) 380–384.
[3] J.T. Brenna, Accounting in Chemical Research 27 (1994) 340–346.
[4] W. Meier-Augenstein, Journal of Chromatography A 842 (1999) 351–371.
[5] H. Craig, Geochimica Cosmochimica Acta 12 (1957) 133–149.
[6] M. Becchi, R. Aguilera, Y. Farizon, M. Flament, H. Casabianca, P. James, Rapid Communication in Mass Spectrometry 8 (1994) 304–308.
[7] S. Horning, H. Geyer, M. Machnik, W. Schänzer, A. Hilkert, J. Oeßelmann, in: W. Schänzer, H. Geyer, H. Gotzmann, A. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, vol. 4, Sport and Buch Strauß, Köln, 1996, pp. 275–283.
[8] R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic, H.G. Pope, Jr., Journal of Mass Spectrometry 31 (1996) 169–176.
[9] R. Aguilera, D.H. Catlin, M. Becchi, A. Phillips, C. Wang, R.S. Swerdloff, et al., Journal of Chromatography B Biomedical Science Applications 727 (1999) 95–105.
[10] U. Flenker, S. Horning, E. Nolteemsting, H. Geyer, W. Schänzer, in: W. Schänzer, H. Geyer, H. Gotzmann, A. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, vol. 6, Sport and Buch Strauß, Köln, 1998, pp. 243–256.
[11] C.H.L. Shackleton, A. Phillips, T. Chang, Y. Li, Steroids 62 (1997) 379–387.
[12] M. Ueki, M. Okano, Rapid Communication in Mass Spectrometry 13 (1999) 2237–2243.
[13] X. de LaTorre, J. Segura, A. Polettini, M. Montagna, Journal of Mass Spectrometry 30 (1995) 1393–1404.
[14] S. Horning, H. Geyer, U. Flenker, W. Schänzer, in: W. Schänzer, H. Geyer, H. Gotzmann, A. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, vol. 5, Sport and Buch Strauß, Köln, 1997, pp. 135–148.
[15] X. De la Torre, J.C. Gonzalez, S. Pichini, J.A. Pascual, J. Segura, Programme and Abstracts of the Manfred Donike Workshop, 17th Cologne Workshop on Dope Analysis, Cologne 14-19 March, 1999, 1999.

USADA 0747

RAPID COMMUNICATIONS IN MASS SPECTROMETRY, VOL. 8, 304–308 (1994)

Gas Chromatography/Combustion/Isotope-ratio Mass Spectrometry Analysis of Urinary Steroids to Detect Misuse of Testosterone in Sport

Michel Becchi,* Rodrigue Aguilera, Yves Farizon, Marie-Magdeleine Flament, Hervé Casabianca and Patrick James

Service Central d'Analyse, CNRS, BP 22, 69390 Vernaison, France

We propose a new confirmatory method for testosterone doping in sport. The present method in use, based on measuring the testosterone/epitestosterone (T/E) ratio in urine, may miss suspicious cases, or lead to reporting cases in which the high ratio is natural. Synthetic testosterone has a ^{13}C abundance different from that of endogenous human testosterone. The connection of a gas chromatograph to an isotope-ratio mass spectrometer via a combustion interface allows the measurement of the corresponding characteristic value (δ‰) for testosterone, its precursors, and its metabolites. To detect exogenous administration of testosterone, 30–40 mL of urine is sufficient.

The use of anabolic steroids was banned by the International Olympic Committee (IOC) for the first time at the Olympic Games in Montreal in 1976. Since that time, athletes have been deterred from the misuse of anabolic steroids by analysis of urine by gas chromatography/mass spectrometry (GC/MS).[1,2,3,4] Testosterone (T) administration is especially difficult to detect because the current method does not distinguish synthetic testosterone from endogeneous testosterone. Detection of testosterone (T) administration is based on the ratio of testosterone to epitestosterone (E) (17 epimer) in urine. According to the IOC, a T/E ratio above 6 constitutes an offence unless there is evidence that this ratio is due to a physiological or pathological condition. Nevertheless, this method fails to distinguish between natural cases where T/E ratios are between 6 and 9 without doping, and cases where T/E ratios are between 4 and 6 because of the end of synthetic T excretion or because of both epitestosterone and testosterone intake.[5] The measurement of the urine ratio of T to luteinizing hormone is helpful but not definitive to detect T administration.[6,7] These ambiguities encouraged us to develop another analytical approach based on isotope ratio mass spectrometry (IRMS). Previous research has shown that the ^{13}C content of endogenous testosterone differs from that of exogenous testosterone and that the difference is significant.[8] The technique used consists of connecting a gas chromatograph to an isotope-ratio mass spectrometer via a combustion interface (GC/C/IRMS).[9] The analysis of the CO_2 molecules ($^{12}CO_2$ and $^{13}CO_2$) allows the calculation of characteristic carbon isotope ratios for each compound.[10] While taking this preliminary work further our preoccupations have been the following:

—to confirm the previous results by analyzing a greater diversity of urine samples;
—to improve the extraction method in order to decrease the urine volume required and make it compatible with doping control samples;
—to analyze steroids from the testosterone metabolic pathways to better distinguish variations of isotopic ratio produced by exogeneous testosterone contribution.

EXPERIMENTAL

Reagents

All the solvents and reagents were of analytical grade purity (SDS, Villeurbanne, France; Prolabo, Vaulx en Velin, France; Janssen, Noisy le Grand, France; Machery-Nagel, Düren, Germany; Merck, Dardilly, France; Aldrich, Saint Quentin, Fallavier, France). Testosterone (T1500), cholesterol (C8667), dehydroepiandrosterone (DHEA, D4000), 5β-androstane-3α, 17β-diol (A7880), and β-glucuronidase from Helix pomatia (G 0876) were supplied by Sigma (Saint Quentin, Fallavier, France).

Solid-phase extractions were carried out on Bakerbond solid-phase extraction (SPE) columns filled with reversed phase octadecyl, J. T. Baker 7020-07, or adsorption-phase silica gel, J. T. Baker 7086-03 (J. T. Baker, Noisy le Sec, France).

The characteristics of the urine samples under study are reported in Table 1.

Table 1. Characteristics of urine samples studied

Urine	Type	T/E	Testosterone administration	Volume used (mL)	Origin
1	Blank	1.0	No	50	P
2	Blank	1.7	No	50	P
3	Blank	1.0	No	100	P
4	E.S.	43	Or	25	P
5	E.S.	49	Or	50	P
6	E.S.	33	Or	50	P
7	E.S.	2.3	Or	50	P
8	E.S.	30	Or	50	P
9	E.S.	20	Or	50	P
10	E.S.	12	I.M.	50	B
11	E.S.	15	I.M.	50	B
12	E.S.	4.6	I.M.	50	B

Blank = urine from drug-free male. E.S. = testosterone excretion study urine from male. T/E = area ratio from steroid GC/MS screen. Or: oral testosterone undecanoate, 2 × 40 mg/day. I.M.: intramuscular testosterone propionate, 50 mg. P: Faculté de Médecine, Pitié Salpétrière, Paris. B: Instituto Municipal de Investigatio Medicale, Barcelona.

* Author for correspondence.

CCC 0951–4198/94/040304–05

USADA 0748

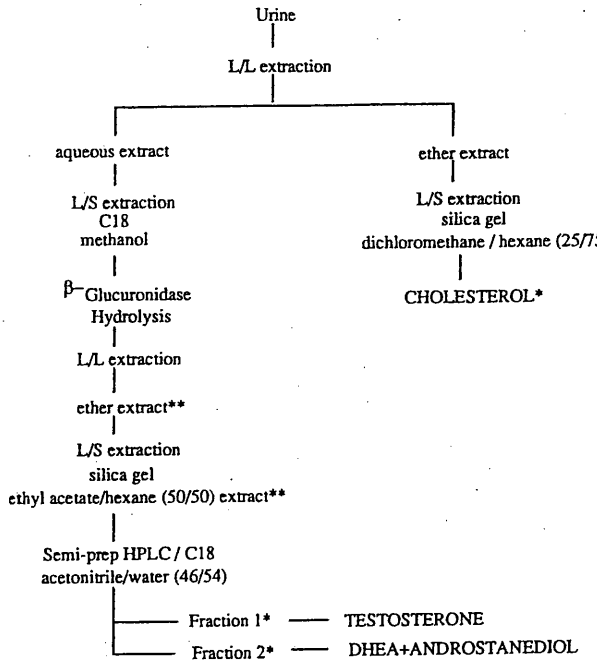


Figure 1. Flow chart of steroids extraction and purification; * GC/EI-MS of acetylated derivatives; ** GC/EI-MS of TMS derivatives.

Preparation of steroids

The flow chart of steroids extraction and purification appears in Fig. 1.

Extraction of free cholesterol. After alkalinization to pH 10 with solid buffer (Na_2CO_3 + $NaHCO_3$, 1:1, w/w), free cholesterol was separated from other conjugated steroids by liquid/liquid extraction with diethyl ether. The cholesterol was recovered from the ether phase by evaporation to dryness while the aqueous phase was kept for conjugated steroid extraction.

Purification of cholesterol. The above dried ether extract was redissolved into a mixture of dichloromethane and hexane (5:95, v/v) then applied to a silica gel SPE column. The cholesterol fraction was eluted with a dichloromethane + hexane mixture (25:75, v/v).

Extraction of conjugated steroids. After cholesterol extraction, the alkaline aqueous layer was first returned to neutral pH and then subjected to SPE extraction on C18 columns (1 g of reverse phase for 25 mL urine) with methanol.

Enzymatic hydrolysis of conjugated steroids. The dried methanolic residue was redissolved in 10 mL of 0.05 M acetate buffer (pH 5.5) and 0.25 mL of β-glucuronidase was added as supplied. Incubation was carried out at 55°C for 3 h.

Extraction of hydrolyzed steroids. After cooling, *ca.* 300 mg of solid buffer (Na_2CO_3 + $NaHCO_3$, 1:1, w/w) were added. Liquid/liquid extraction was performed with diethyl ether (2 × 50 mL).

First step of deconjugated steroids purification: silica gel. The ether extract of hydrolyzed steroids was redissolved in an ethyl acetate + hexane mixture (5:95, v/v) and applied to a silica gel SPE column. Steroid elution was obtained with an ethyl acetate + hexane mixture (50:50, v/v).

Second step of deconjugated steroids purification: semi-preparative high-performance liquid chromatography (HPLC). The dried extract from silica-gel purification was redissolved in 25 µL of methanol and injected on a semi-preparative C18 column: Spherisorb ODS2 (Interchim; 25 cm × 1 cm; 5 µm). The mobile phase, water + acetonitrile (54:46, v/v), was delivered by a Merck L-600 pump at a flow rate of 4 mL/mn. Elution was isocratic and was monitored by UV detection (Kratos (Manchester, UK) Spectroflow 757 at 240 nm). Testosterone was obtained in the 13 to 15 mL volume range (fraction 1), and the DHEA (dehydroepiandrosterone) and androstanediol mixture was collected between 16 and 19 mL (fraction 2).

Acetylation of steroids. Acetylation of the purified extracts was carried out by overnight room temperature incubation with a mixture of pyridine and acetic anhydride (200 µL of each). Acetylated steroids were then dried in a Speedvac concentrator (Bioblock Scientific, Illkirch, France).

GC/EI-MS analysis. GC/EI-MS analyses were performed on a Hewlett-Packard (HP) 5970B mass-selective detector linked to an HP 5890 gas chromatograph equipped with an HP Ultra-1 (100% methyl silicone) fused-silica capillary column (17 m × 0.2 mm i.d.; 0.33 µm film thickness). The injections were made in the splitless mode (1 to 2 µL). The carrier gas was helium.

The trimethylsilyl (TMS) derivatives were analyzed as already described.[1,2,3] For acetylated derivatives the oven temperature started at 200°C (1 min), increased at 2°C/min to 250°C, then at 20°C/min to 300°C and stayed at the final temperature for 4 min. For cholesterol acetate, the temperature increased from 200°C to 300°C at 15°C/min.

Isotopic analysis

GC/C/IRMS analyses were performed on an HP 5890 gas chromatograph connected to a home-made combustion furnace (Centre National de la Recherche Scientifique—Service Central d'Analyse) linked to a Finnigan MAT (Bremen, Germany) 252 isotope ratio mass spectrometer. The GC was equipped with an HP-1 column (Hewlett-Packard; 100% methyl silicone; 25 m × 0.32 mm). Helium was the carrier gas (20 psi). Samples were injected in the splitless mode at 285°C. The GC separation began at 80°C and the temperature increased to 280°C at a rate of 10°C/min.

The temperature of the combustion oven was 800°C and the oxidative catalyst was a wire of copper oxide (0.25 mm in quartz tubing of 0.5 mm). The following alkanes were used as internal standards: C_{16}, C_{18}, C_{20}, C_{22}, C_{24} and C_{28} (Table 2).

The δ standard notation for expressing carbon isotope ratios is defined as the relative difference in isotope ratio between the sample and an international standard (PDB or Pee Dee Belemnite), calculated as:

$$\delta^{13}C‰ = \left(\frac{R_{sample} - R_{PDB}}{R_{PDB}}\right) \times 10^3$$

Table 2. ^{13}C δ‰ of standard paraffins used

Paraffins	δ‰	σ
C_{16}	−50.97	0.05
C_{18}	−26.61	0.07
C_{20}	−43.95	0.11
C_{22}	−34.03	0.08
C_{24}	−32.02	0.10
C_{28}	−26.43	0.16

where R_x refers to the $^{13}C/^{12}C$ ratio of the sample or international standard. A typical standard deviation associated with these GC/C/IRMS determinations is 0.4‰.

RESULTS AND DISCUSSION

This work included two main steps: extraction and purification of urinary steroids, and actual isotopic analysis.

The first difficulty that we set out to overcome was the limited volume of urine available. Preliminary work[8] required 200–400 mL of urine. In the doping control context, volumes much greater than 50 mL are simply not available. Fortunately, new generations of isotope ratio mass spectrometers interfaced with gas chromatographs reach detection thresholds in the 10 ng range under optimal conditions, allowing δ‰ measurements of low-level organic compounds. Taking advantage of this improved sensitivity, we worked with urine volumes of about 15–50 mL. For urines with relatively high testosterone concentration ([T] ≥ 100 ng/mL), 30–40 m seemed to be sufficient.

In addition to testosterone, we conducted isotopic ratio measurements on testosterone precursors cholesterol and DHEA, and on testosterone metabolite 5β-androstane-3α, 17β-diol. We reasoned that the isotopic ratio in the precursors should not be affected by exogenous testosterone contribution, but would reflect the natural isotopic ratio for the individual, and in fact constitute a kind of 'autostandard', characteristic of each individual (Fig. 2). In contrast, the metabolite may reflect a change in isotopic ratio following exogenous testosterone administration. By including precursors and metabolites in our study we hoped to discover indicators of testosterone administration perhaps better than testosterone itself.

Steroid extraction and purification (Fig. 1) first yielded urinary cholesterol (C) by diethyl ether extraction (see Fig. 3(a)). Fraction 1 from C_{18} HPLC (see Fig. 3(b)) contained testosterone (T) and other compounds, and fraction 2 (see Fig. 3(c)) contained the DHEA (D) and 5β-androstane-3α, 17β-diol (A) mixture.

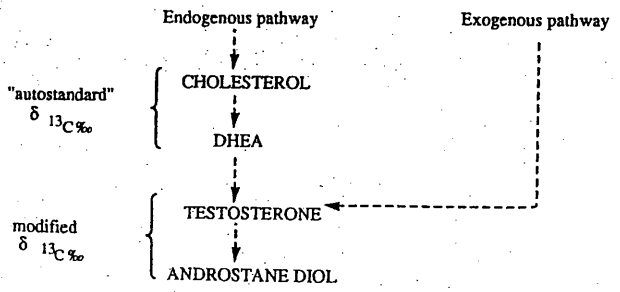


Figure 2. Investigated steroids from the androgens metabolic pathway.

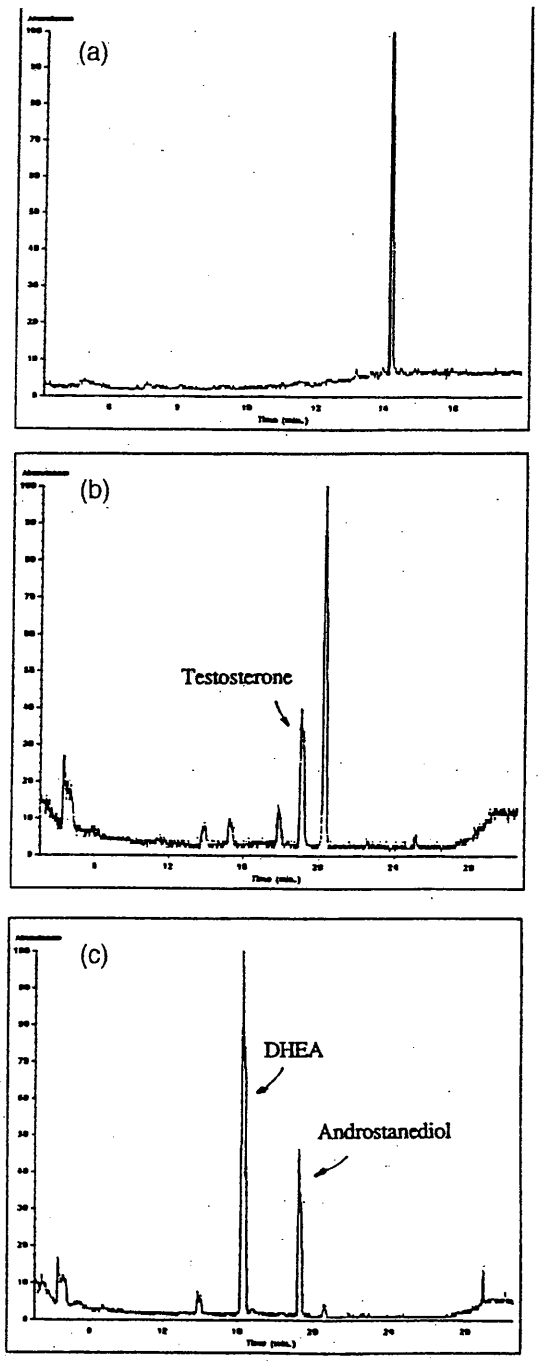


Figure 3. GC/EI-MS control total ion currents of acetylated steroids before GC/C/IRMS. (a) Cholesterol fraction, (b) HPLC fraction 1 and (c) HPLC fraction 2.

At each main step we checked recovery by GC/EI-MS on trimethylsilylated (TMS) or acetylated derivatives[1,2,3] (Fig. 1). TMS derivatives form faster than acetylated derivatives, which expedites the work, and their mass spectra are richer; however silicon is not compatible with the combustion step of GC/C/IRMS. As in the previous published work,[8] we conducted IRMS on acetylated derivatives. These are remarkably stable and have good chromatographic properties. Only hydroxyl groups (not enols) get derivatized, so that in the example of testosterone, only 2 carbon atoms are added to the original 19. Since the measurement of the carbon isotopes was of the essence, there was an advantage in selecting a derivatization that would add few carbons and therefore barely modify the

Table 3. Urine sample results of δ‰ measurements

Urine samples	Steroids δ‰ Cholesterol (C)	DHEA (D)	Testosterone (T)	Androstanediol (A)	Differences in δ‰ T−C	T−D	A−C	A−D
1	−24.5	−23.8	−26.1	−27.1	1.6	2.3	2.6	3.3
2	−24.8	−24.6	−26.0	−26.7	1.2	1.4	1.9	2.1
3	−25.3	−23.9	−26.3	−27.2	1.0	2.4	1.9	3.3
4	−24.0	−24.2	−30.8	−31.0	6.8	6.6	7.0	6.8
5	−24.5	−24.9	−29.4	−30.7	4.9	4.5	6.2	5.8
6	−25.4	−26.0	−30.8	−30.0	5.4	4.8	4.6	4.0
7	−24.6	−24.4	−28.3	−30.8	3.7	3.9	6.2	6.4
8	−24.5	−23.8	−28.3	−31.0	3.8	4.5	6.5	7.2
9	−25.3	−24.1	−28.5	−31.5	3.2	4.4	6.2	7.4
10	−24.0	n.a.	−27.7	−31.9	3.7	n.a.	7.9	n.a.
11	−24.3	n.a.	−28.4	−29.0	4.1	n.a.	4.7	n.a.
12	−23.8	−22.9	−28.3	−27.5	4.5	5.5	3.7	4.7

n.a. = not available.

isotopic ratio of the molecule under study. All compounds of interest were identified as acetylated and trimethylsilylated derivatives by comparison to authentic reference standards. The GC/EI-MS chromatograms of the acetylated fractions submitted to GC/C/IRMS analysis appear in Fig. 3. During the steroid preparation we maximized recovery at the expense of purity. This is because the ^{12}C and ^{13}C homologs of a given compound may get partially resolved, so that excessive fractionation may lead to artefactual enrichment in one or the other isotope (isotopic discrimination).

First of all, we controlled the influence of acetylation on steroids IRMS. Aliquots of testosterone were treated with different quantities of acetic anhydride and pyridine. The results ($\delta‰ = -30.2 \pm 0.4$) indicated that acetylation was not discriminatory towards isotopic ratio measurement.

The results of the isotopic measurements for standard paraffins appear in Table 2, and those for the steroids in each urine appear in Table 3. For urine samples 10 and 11, the low volume of urine available on the one hand and/or the loss of compounds during purification on the other hand, precluded analysis for DHEA. As expected, precursors' δ‰ values were not affected by exogenous testosterone administration. On the other hand, for testosterone and its metabolite, δ‰ values were clearly decreased. With regard to testosterone, our results (Table 3) appear comparable to those of Southan *et al.*[8] (blank urine δ‰ testosterone $= -26.6 \pm 0.25$; excretion study urine δ‰ testosterone $= -30.4 \pm 0.42$). Between blank urines and excretion-study urines, δ‰ testosterone values [and δ‰ 5β-androstane-3α, 17β-diol values] were significantly different (Student t-test $T = 7.2109$, $p = 0.0001$) [and $T = 4.1238$, $p = 0.0021$, respectively]. We noticed that post administration urine sample 7 with a T/E ratio of only 2 had a large difference of δ‰ values between (C or D) and (T or A).

We calculated four new variables, namely the differences in δ‰ between T, or else metabolite A on the one hand, and precursor C or D on the other hand, and referred to them as T − C, T − D, A − C, A − D. Next we compared blank urines and excretion-study urines according to these four new variables and found all four sets of differences to be significant, with the clearest differences for T − C and A − C, most likely due to the availability of nine (for C) rather than only seven (for A) users' data points.

Choosing the two new variables with the most significant differences, we plotted Fig. 4 showing (δ‰ testosterone − δ‰ cholesterol) as a function of δ‰ androstanediol − δ‰ cholesterol).

The urines investigated fall into two classes:

—blank urines,
—testosterone excretion-study urines.

Two populations became evident in Fig. 4: one

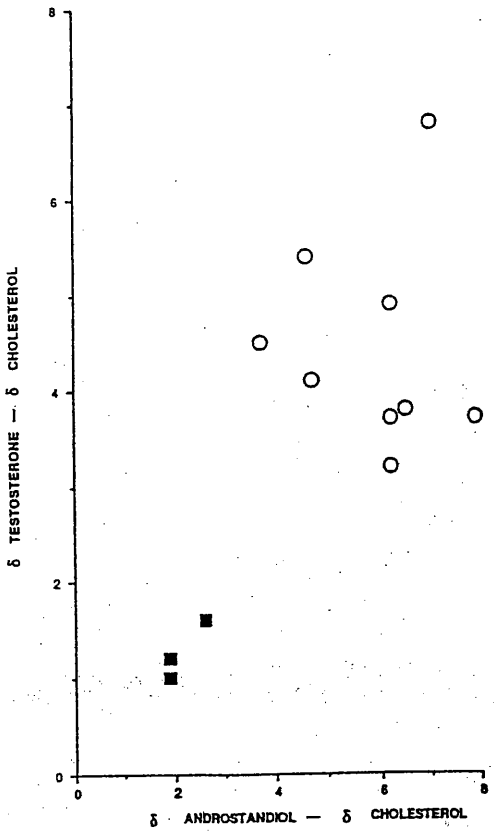


Figure 4. δ‰ differences between testosterone and precursor cholesterol as a function of δ‰ differences between androstanediol and precursor cholesterol, for the urine samples investigated. Points marked ○ correspond to testosterone from excretion-study urine and those marked ■ from blank urine.

corresponding to endogenous natural testosterone, and another where exogenous administration took place. For blank urines, there were few variations of δ‰ values among the different representatives of the metabolic chain.

CONCLUSION

This method of analysis holds great promise for resolving cases where testosterone doping is suspected. It allows classification of athletes' urines into 2 catagories:

—undoped: natural ^{13}C δ‰ testosterone not greater than about −27‰
—doped: ^{13}C δ‰ testosterone greater than about −27‰.

The volumes of urine required are in agreement with volumes normally collected for doping control. However, to validate the present GC/C/IRMS method, it would be necessary to investigate a greater number of urine samples from different ethnic groups and from subjects with different dietary habits, among other things. This would be possible with the collaboration of other IOC-accredited doping-control laboratories.

Acknowledgements

We thank Dr F. Wright (Faculté de Médecine, Pitié Salpétrière, Paris, France) and Prof. G. Segura (IMIM, Barcelona, Spain) who kindly provided us with urine samples. We gratefully acknowledge Dr C. K. Hatton (Olympic Analytical Laboratory, Los Angeles, USA) for useful discussion and comments during manuscript preparation. This work was supported in part by the Région Rhône Alpes grant.

REFERENCES

1. C. K. Hatton and D. H. Catlin, *Clinics in Lab. Medicine* **7** 655 (1987).
2. R. Masse, C. Ayotte and R. Dugal, *J. Chromatogr.* **489** 23 (1989).
3. B. Chung, H.-Y. Choo, T. Kim, K. Eom, O. Kwon, J. Suh, J. Yang and J. Park, *J. Anal. Toxicology* **14** 91 (1990).
4. W. Schanzer and M. Donike, *Anal. Chim. Acta* **275** 23 (1993).
5. D. H. Catlin and C. K. Hatton, *Adv. Intern. Med.* **36** 399 (1991).
6. A. T. Kicman, R. V. Brooks, S. C. Collyer, D. A. Cowan, M. N. Nanjee, G. J. Southan and M. J. Wheeler, *J. Sports Med.* **4** 253 (1990).
7. R. V. Brooks, G. Jeremiah, W. A. Weeb and M. Wheeler, *J. Steroid Biochem.* **11** 913 (1979).
8. G. Southan, A. Mallet, J. Jumeau, S. Craig, N. Poojara, D. Mitchell, M. Wheeler and R. V. Brooks, Programme and Abstracts of the Second International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona 1990, p. 306.
9. D. E. Matthews and J. M. Hayes, *Anal. Chem.* **50** 1465 (1978).
10. H. Craig, *Geochim. and Cosmochim. Acta* **12** 133 (1957).

DJB 10-8-97

ELSEVIER

Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: Potential use in gas chromatography isotope ratio mass spectrometry

Cedric H.L. Shackleton,* Esther Roitman,* Andy Phillips,† and Tony Chang*

**Children's Hospital Oakland Research Institute, Oakland, California, USA; and †Micromass, Wythenshawe, Manchester, United Kingdom*

The basis of a potential method for confirming intake of four natural androgens (testosterone, epitestosterone, dihydrotestosterone, and dehydroepiandrosterone is presented. The method relies on isolating from urine a steroid fraction containing androstenediol and androstanediol metabolites of these natural steroids and analyzing their ^{13}C content by gas chromatography, combustion, isotope ratio mass spectrometry. The steroids were recovered from urine by conjugate hydrolysis with a Helix pomatia *preparation (sulfatase and β-glucuronidase), Girard T reagent separation to obtain a nonketonic fraction, and Sephadex LH-20 chromatography for purification. Metabolites appropriate for all of the natural steroids could be separated (as diacetates) by gas chromatography on a DB-17 capillary column viz.: 5α (and β)-androstane-3α,17α-diol (epitestosterone as precursor); 5α (and β)-androstane-3α,17β-diol (testosterone as precursor); 5-androstene-3β,17β-diol (dehydroepiandrosterone precursor); and 5α-androstane-3α,17β- (and 17α-) diol (dihydrotestosterone precursor). Measurement of the ^{13}C content of the specific analytes after ingestion of the androgen precursors demonstrated a lowering of $\delta^{13}C‰$ value compared to normal values. Typically, in the male individual studied, $\delta^{13}C‰$ values for all components were −26 to −27 before drug administration and −29 to −30 at 6 h after, the latter values reflecting those obtaining for commercial synthetic steroid compared to in vivo synthesized steroid. While generally the metabolism of the steroids was as expected, this was not the case for 5α-dihydrotestosterone. A major metabolite was 5α-androstane-3α,17α-diol, which had presumably been formed by 17β/17α isomerization, a process previously known for unnatural anabolics but not for natural hormones. The isolation, purification, and isotope ratio mass spectrometry techniques described may form the basis of a general method for confirming natural steroid misuse by sports participants.* (*Steroids* **62**:665–673, 1997) © *1997 by Elsevier Science Inc.*

Keywords: gas chromatography combustion/isotope ratio mass spectrometry; doping; androgen; dihydrotestosterone; dehydroepiandrosterone; epitestosterone

Introduction

Recent communications by Southan et al.,[1] Becchi et al.,[2] Aguilera et al.,[3] Horning et al.,[4] and ourselves[5] addressed the use of isotope ratio mass spectrometry (IRMS) to detect testosterone (T) misuse in sports. Testosterone commercially synthesized from plant precursors has a lower ^{13}C content, reported as the $\delta^{13}C‰$ value,[2] compared to endogenously produced steroid. This is because synthetic testosterone tends to be made from a plant species (usually soy) with low ^{13}C content ($\delta^{13}C‰ < -30$) while our bodies (and their chemical products) originate from a variety of plants that are a mixture of relatively high ($\delta^{13}C‰$ −12 to −20) and low ($\delta^{13}C‰$ −23 to −35) values.[6] Typical values for human endogenous steroid products measured free or as acetate derivatives by different investigators[1–5] give $\delta^{13}C‰$

Address reprint requests to Cedric H.L. Shackleton, Children's Hospital Oakland Research Institute, 747 52nd Street, Oakland, CA 94609, USA. Tel.: (510) 428-3604; Fax: (510) 428-3608; E-mail: shckltn@lanminds.com.
Received April 22, 1997; accepted May 28, 1997.

Steroids 62:665–673, 1997
© 1997 by Elsevier Science Inc. All rights reserved.
655 Avenue of the Americas, New York, NY 10010

0039-128X/97/$17.00
PII S0039-128X(97)00065-2

USADA 0753

values ranging from −24 to −28. The publications of groups who have worked in this field suggest that values of around −29 and below for urinary testosterone and its metabolites is conclusive evidence of exogenous testosterone administration.[1–5]

Testosterone is not the only natural androgen-like compound used by athletes. Others include epitestosterone (epiT), dehydroepiandrosterone (DHEA), and dihydrotestosterone (DHT). While epiT is not androgenic, it has been administered to mask the results of the commonly used T/epiT ratio. The greater than 6 value for the ratio adopted as proof of misuse[7] can be normalized by self-administering epiT as well as testosterone. DHT, the 5α-reductase-produced metabolite of testosterone, is itself a potent androgen which has in recent years been shown to be the subject of misuse.[8] DHEA oral administration is an interesting problem. While it has been banned by the International Olympic Committee (IOC), it is freely available at low cost in the United States marketed as a "nutritional supplement." The health and anti-aging properties of this steroid, although less than well proven, are being widely touted. DHEA itself is a weak androgen and only a minimal amount, probably ≤1%, is converted to testosterone. While not even a C_{19} steroid, pregnenolone (in pill form) is also freely available in the United States and is being recommended for daily use by elements of the "health food industry." Pregnenolone has not been banned by the IOC but this may be anticipated if its ingestion is perceived to contribute to androgen synthesis.

Our previous study demonstrated the use of IRMS for confirming testosterone administration by measuring the ^{13}C content of its metabolites 5α- and 5β-androstane-3α,17β-diols. While these are not major steroid components, accounting for only about 4% of total testosterone metabolites,[9] they are structurally closely related to the precursor, particularly in contrast to the quantitatively important metabolites androsterone and etiocholanolone. Not only that but it is believed that in humans they are not formed to a significant extent by reduction of 17-oxosteroids.[9] The other natural steroids potentially misused also metabolize to androstane- or androstenediols (Figure 1), probably to a similar extent as does testosterone (3–5%), although diols are probably relatively more important in epitestosterone metabolism since little of this steroid is oxidized by 17α-hydroxysteroid dehydrogenase to 17-ketosteroids. Our previously described methodology[5] has been refined to encompass all of these metabolites, thus allowing comprehensive evaluation of natural hormone misuse through conducting a single gas chromatography (GC)/combustion/IRMS analysis. While this has been a pilot study, we believe that with further development and evaluation the methodology will be useful for routine screening use and may replace the T/EpiT ratio and other parameters currently used in doping control.

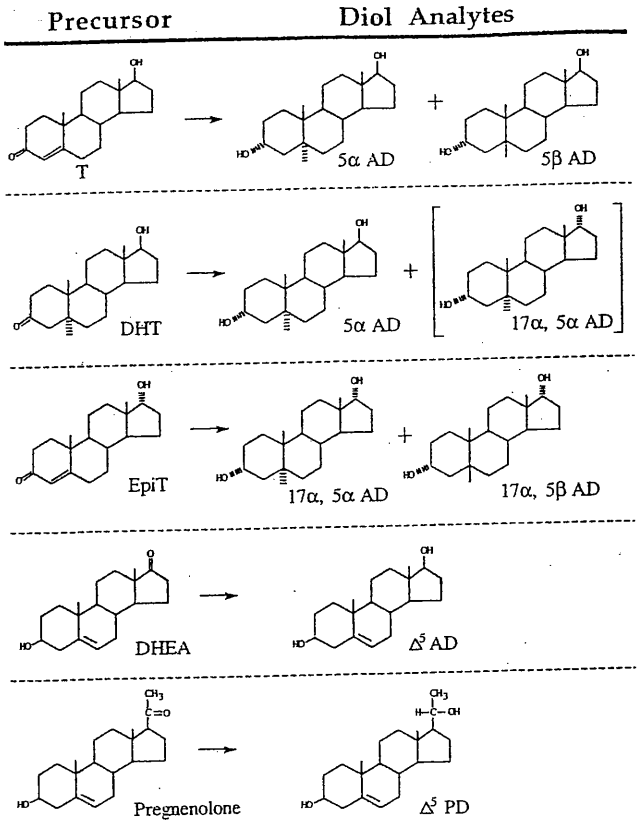


Figure 1 Diol metabolites of androgens and pregnenolone. The following abbreviations are used: 5αAD, 5α-androstane-3α,17β-diol; 5βAD, 5β-androstane-3α,17β-diol; 17α,5αAD, 5α-androstane-3α,17α-diol; 17α,5βAD, 5β-androstane-3α,17α-diol; Δ^5AD, 5-androstene-3β,17β-diol; and Δ^5PD, 5-pregnene-3β,20α-diol. 5α-Androstane-3α,17α-diol as a DHT metabolite is still controversial, so it has been bracketed. Not shown are the minor but significant testosterone, DHT, or epiT metabolites 5α-androstane-3β,17β (and 17α)-diols.

Experimental

Materials

DHT, epitestosterone, androstanediols, androstenediols, and other authentic steroids were obtained from Sigma Chemical Company (St. Louis, MO). DHEA and pregnenolone in "dietary supplement" tablet form were marketed by Natron (Chatsworth, CA) and Optimal Nutrients (Foster City, CA), respectively. The $\delta^{13}C‰$ values of acetates of these reference steroids were between −30 and −32. All solvents were of analytical grade.

Urine sample analysis

A representative urine sample was prepared for method evaluation. It was composed of an equal proportion of daily 24-h samples of urine from 15 male and 15 female individuals.

In addition, steroid metabolites excreted by a male adult after steroid ingestion were studied. This individual gave written informed consent. In separate experiments conducted 2 weeks apart, 50 mg of testosterone, DHT, epiT, DHEA, and pregnenolone were taken orally for 2 days. Urine was voided 3 and 6 h after the second administration, but only the second sample was collected and analyzed. In addition, collections were made 3 and 6 h after co-administration of 50 mg of testosterone and 5 mg of epitestosterone. The 6-h time period was chosen because Schänzer et al.,[10] found maximal excretion of androstanediols at that period after steroid ingestion.

USADA 0754

Preparation of steroid extracts for GC/combustion/IRMS

The basic methodology was described previously although modifications have been made for simplicity.[5] The principal change has been deletion of a bismuthate oxidation step. In brief, 30 ml of urine were extracted by Sep-pak® [11] and hydrolyzed by *Helix pomatia* enzymes. Whereas in the method reported previously,[5] Girard separation was undertaken on the hydrolysate directly, in this study the steroids were first extracted by Sep-pak cartridge. To the steroid extract was added 0.5 ml of acetic acid and 200 mg of Girard reagent T (Sigma Chemical Company, St. Louis, MO). The reaction was allowed to proceed for 30 min at 100°C whereupon the reaction tube was cooled and low polarity nonketonic steroids extracted with 2 × 3 ml of isooctane/methylene chloride (2:1). The extract was washed with 0.5 ml of 10% NaOH, and 0.5 ml of water and finally dried with a few milligrams of anhydrous sodium sulfate. Once dried, the steroid extract was purified on mini-Sephadex LH-20 columns (Pharmacia Biotech AB, Uppsala, Sweden) prepared in glass wool-plugged Pasteur pipettes (dry mass 0.5 g, bed size 60 × 5 mm inside diameter). The Sephadex LH-20 was stored in the solvent system (cyclohexane/ethanol, 4:1) to allow it to maintain a swollen state. The extract was applied in 0.2, 0.2, and 0.4 ml of the solvent system, allowing each application to absorb into the bed. Another 1 ml of solvent was added directly to the absorbent bed, and all of this initial eluant (1.8 ml) was discarded. The nonketonic androstanediol fraction was recovered by eluting the column with 1.4 ml of the same solvent. The steroid-containing fraction was dried and following addition of 1 μg of 5α-androstan-3β-ol internal standard was acetylated overnight with 100 μl of pyridine and 200 μl of acetic anhydride. The derivative was dried, dissolved in cyclohexane, and analyzed by GC/mass spectrometry (MS) and GC/combustion isotope mass spectrometry.

Gas chromatography/mass spectrometry

Authentication of the structures of the steroids present in the steroid fraction was achieved by GC/MS utilizing an HP 5971 MSD instrument (Hewlett-Packard, Palo Alto, California, USA) with a DB-17 column (30 m, 0.25 mm inside diameter, 0.25 μm film thickness; J&W Scientific, Folsom, California, USA).

For separation of the steroid acetates the following instrument conditions were utilized: injector 260°C, interface 280°C, splitless injection. Program: after injection at 50°C a 1-min hold, then 30°C/min to 270°C and 0.5°C/min to 282°C. Mass spectra were obtained by scanning a 50–650-amu range. Component structures were confirmed by comparing urinary and authentic steroid spectra. Molecular ions of the androstanediols and androstenediols were small but major ions were formed by —OAc removal (M-60 and M-60-60).

Gas chromatography/combustion/isotope ratio mass spectrometry

This was performed on a Micromass isochrom isotope ratio mass spectrometer (Micromass UK Ltd., Wythenshawe, Manchester, UK) as previously described.[5] Steroid separations were conducted on a DB-17 column identical with that used for GC/MS. Analysis of the steroid containing fraction took 20 min. ^{13}C isotope content (measured as $\delta^{13}C‰$) was determined for androstanediols and androstenediols and the endogenous reference compounds (ERCs), pregnanediol, and pregnanetriol. In our previous communication we used pregnanediol as the ERC, but pregnanetriol has an advantage because it is in a region of the chromatogram less subject to contamination and larger amounts (than pregnanediol) are excreted in adult males and in women during the greater part of their menstrual cycles.

Results and discussion

The analytes and their separation

Androstanediol and androstenediol metabolites have for some years been included in studies on the detection of endogenous steroids.[12–15] We have detected three androstenediols and six androstanediols in urine extracts (Table 1). The probable origins of these steroids are as follows: 5-androstene-3β,17β-diol is a metabolite of DHEA; 5-androstene-3β,17α-diol may be a side product of conversion of pregnenolone to 17-deoxysteroids[16] or be formed by 17α-hydroxysteroid dehydrogenase acting on DHEA; 5β-

Table 1 Retention indices and relative quantitative importance of urinary androstanediol and androstenediol components

Steroid	RI	Relative importance (% total)		
		HP	βGlucA	βGlucB
5α-Androstane-3β-ol (IS)	2866			
Androstenediol (unident.)	3132	10.0	9.7	NM[c]
5β-Androstane-3α,17α-diol	3192	7.3	11.1	1.0
5β-Androstane-3α,17β-diol	3217	32.0	33.1	52.0
5α-Androstane-3α,17α-diol	3230	4.7	3.1	10.0
5α-Androstane-3α,17β-diol	3241	14.0	16.8	15.7
5-Androstene-3β,17α-diol	3279	8.7	23.8	20.2
5α-Androstane-3β,17α-diol	3285	1.0	2.2	NM
5-Androstene-3β,17β-diol	3293	22.0	1.3	1.4
5α-Androstane-3β,17β-ol	3300	~1.0	~1.0	~1.0
Pregnanediol (ERC)	3406			
5-Pregnene-3β,20α-diol	3483			
Pregnanetriol (ERC)	3645			

Retention times and approximate proportions of androstanediols and androstenediols in urine extracts produced in this study by *H. pomatia* (HP) and β-glucuronidase hydrolysis (β-Gluc A). Results are also presented for Dehennin's data also obtained using β-glucuronidase hydrolysis (β-Gluc B). The values given assume a total of 100%. IS, internal standard; ERC, endogenous reference compounds.

USADA 0755

androstane-3α,17β-diol and 5α-androstane-3α,17β-diol are testosterone metabolites and the latter is also a DHT metabolite; 5α-androstane-3β,17β-diol is a DHT and testosterone metabolite; epitestosterone gives rise to 5α (and 5β)-androstane-3α,17α-diols and 5α-androstane-3β,17α-diol.

Separation of all these steroids from urine as diacetate derivatives is shown in Figure 2, and the chromatogram can be considered average because the sample was prepared from equal proportions of 24-h urine collections from 15 male and 15 female adults. The chromatogram, the best that could be achieved within a practical time (30 min), shows that almost all steroids can be baseline separated. The earliest eluting androstenediol has not been identified although it was shown not to be 5-androstene-3α,17β-diol, 4-androstene-3β,17α (or β) -diol, or 4-androstene-3α,17α (or 17β) diols. 5-Pregnene-3β,20α-diol is a pregnenolone metabolite and the ERCs pregnanediol and pregnanetriol are metabolites of progesterone and 17-hydroxyprogesterone, respectively. Regarding the elution properties of the group, the following elution order applies: 3α-hydroxy-5β, 3α-hydroxy-5α, 3β-hydroxy-Δ^5, and 3β-hydroxy-5α. Superimposed on this order 17α-hydroxylated steroids elute before 17β.

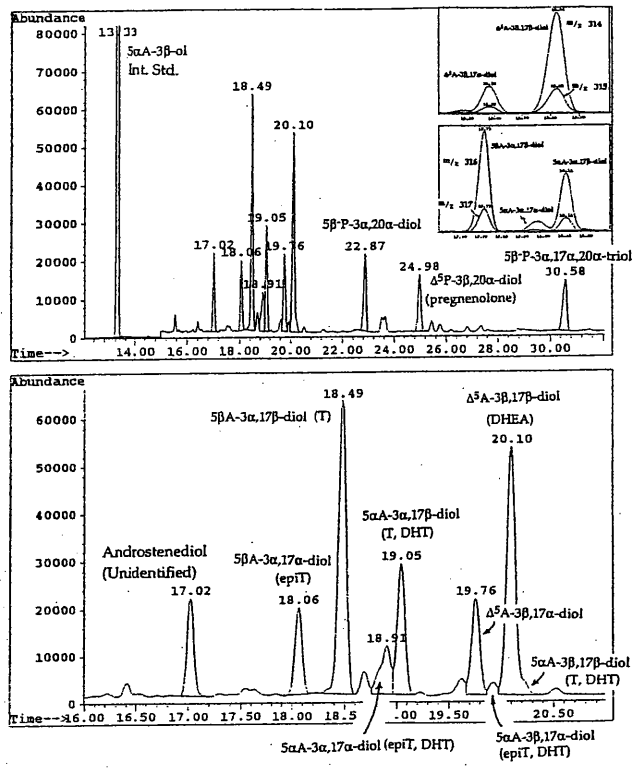


Figure 2 The contents of the diol fraction of normal urine. The upper panel shows the second half of the full steroid diacetate chromatogram including the internal standard and ERCs. The lower panel focuses on the androstanediols and androstenediols. The hormonal precursors of the individual metabolites are listed in parentheses. The inset shows selected ion monitoring results of M-60 and M-60 + 1 for the androstenediols and androstanediols, indicating that ^{12}C- and mono-^{13}C-labeled steroids exactly co-eluted.

Table 1 gives the retention indices of the androstanediols and androstenediols. The approximate amount of each metabolite (obtained after *H. pomatia* hydrolysis) relative to the total is also given and compared to values we obtained using *Escherichia coli* β-glucuronidase and those reported by Dehennin,[13] who also used the *E. coli* preparation. The obvious difference between β-glucuronidase and *H. pomatia* results is that 5-androstene-3β,17β-diol is a minor component of the β-glucuronidase profile, representing about 1/15 of the 17α-epimer. With *H. pomatia* hydrolysis, the 17β-epimer dominates reflective of its largely sulfate conjugation. The results and those of Dehennin[13] broadly agree; an exception is the low excretions of 5β-androstane-3α,17α-diol found in the latter study. The pregnenolone metabolite 5-pregnene-3β,20α-diol, an important component found after *H. pomatia* hydrolysis, was essentially absent from extracts hydrolyzed with β-glucuronidase. The comparative enzyme studies confirmed the advantage of using *H. pomatia* mixed β-glucuronidase and sulfatase for the current studies.

A desirable feature of the GC separation on the DB-17 column was that there was no apparent isotope fractionation of the intact steroids that would compromise IRMS measurement. The inset in Figure 2 shows *m/z* 316 and 317 peaks (M-60 and M-60 + 1) for androstanediols and *m/z* 314 and 315 for androstenediols, and it is quite evident that diol molecules with one ^{13}C label are not even partially resolved from completely ^{12}C-containing steroids.

Steroid excretion and δ% values after DHT administration

It is evident from the chromatogram illustrated in Figure 3 that two steroids, 5α-androstane-3α,17β-diol and 5α-androstane-3α,17α-diol, are dramatically increased in size relative to normal values (cf. Figure 2). Although increase in the former metabolite was expected, this was not the case for the 17α-diol, partly because its production from the 17β-diol seemed unlikely and partly because it has not previously been reported as 5αDHT metabolite.[8,10–15] Evi-

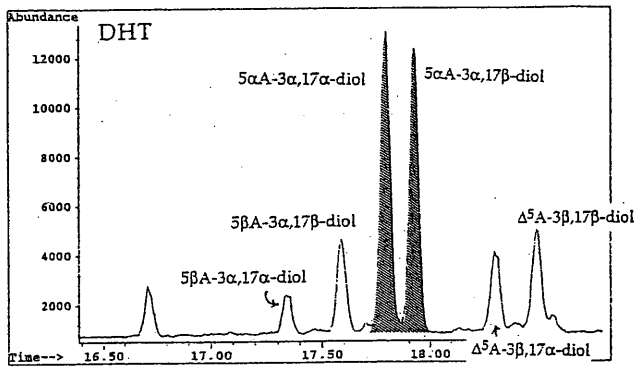


Figure 3 The steroid fraction following DHT administration. Only the central part of chromatogram is illustrated. The major metabolites are highlighted and were characterized as 17α- and 17β-androstanediol epimers. In contrast to undrugged state and to the situation following testosterone administration, the ratio of the amount of 5α-androstane-3α,17β-diol to 5β-androstane-3α,17β-diol is reversed.

USADA 0756

dence that both compounds were metabolites of ingested 5αDHT comes from the fact that they both have lowered IRMS δ values (Table 2) whereas normal values (~ −26) were obtained for 5β-androstane-3α,17β-diol, 5-androstene-3β,17α-diol, and 5-androstene-3β,17β-diol. The finding that a 17α-androstanediol is a significant metabolite (at least in the individual studied) suggests that 17-hydroxyl isomerization is an important feature of 5αDHT metabolism. Quantitatively, and from the δ measurement (Table 2), it is unlikely that 5α-androstane-3α,17α-diol could have originated by serial activity of 17β-(oxidative) and 17α-hydroxysteroid (reductive) dehydrogenase on DHT. Previous studies on testosterone and nandrolone (19-nor-testosterone) have shown little if any reduction of 17-oxo-steroid metabolites following their production.[17–19] Although human 17β/17α isomerization has previously been reported for synthetic anabolics, including trenbolone, which has a conventional (not 17α-methylated) 17β-hydroxy D-ring,[20–22] this form of transformation has not been reported in natural hormone metabolism. Confirmation that isomerization must have occurred comes from an experiment (to be separately reported) where 7 mg of [16,16,17-$^2H_3$]DHT were administered and androstanediol metabolites were isolated. Both 5α-androstane-3α,17β-diol and 5α-androstane-3α,17α-diol metabolites had major components retaining three deuteriums. Although production of a 17α-hydroxylated product was significant, it must be borne in mind that it is still a quantitatively minor metabolite since the total amount of androstanediols represents only 10% of the urinary metabolites found after DHT ingestion.[10]

Epitestosterone administration

Although there are no IOC procedural guidelines governing epiT administration to athletes, it has been recommended that excretions >150 μg/liter should be further investigated. Such high excretions together with lowered δ value of metabolites may well provide definitive proof of misuse.

In our experiment, separate analysis of the total steroid fraction (before Girard separation) by GC/MS indicated that a large proportion of epitestosterone was excreted unchanged (with possible exception of conjugation) as reported in other studies.[14] This was expected because the relatively low activity of 17α-hydroxysteroid dehydrogenase in humans would not contribute to androstenedione and its metabolites androsterone and etiocholanolone greatly. Although 17-oxidation was not substantial, the excretion of androstanediol metabolites isolated after a 50-mg epiT administration was significant, and the chromatogram contained large peaks with retention times appropriate for 5β-androstane-3α,17α-diol, 5α-androstane-3α,17α-diol, and a lesser amount of 5α-androstane-3β,17α-diol (Figure 4). Isotope ratio measurement of the first two peaks showed they had highly negative δ values. The large amounts of 17α-androstanediols present in this experiment rendered the measurement of δ values for the other androstanediol and androstanediol compounds difficult and inaccurate, but they are of course likely to be normal. The amount of epiT administered was excessive considering its natural production rate and would not represent a typical amount taken by an athlete attempting to evade a positive T/epiT ratio. A secondary experiment was performed with a better model study, and chromatograms in Figure 4 (lower panel) show separation of steroids following a 50-mg testosterone administration and 6 h after a combination of 50 mg of testosterone and 5 mg of epitestosterone. When epitestosterone is taken in sports it is taken only to normalize the T/epiT ratio in cases where T is being administered. Thus the amount taken of epiT would be small compared to that of testosterone, particularly since epiT resists metabolism. An increase in the relative peak size of the 17α-androstanediols after T + epiT administration was evident but IRMS measurement was not conducted, so it is not known whether drug administration at this level could be confirmed by the isotope ratio technique.

DHEA administration

DHEA is rapidly metabolized by reduction to 5-androstene-3β,17β-diol or conversion to androsterone and etiocholanolone. A chromatogram of metabolites excreted after DHEA ingestion is shown in Figure 5 and illustrates a major increase in 5-androstene-3β,17β-diol peak size relative to the other constituents, and IRMS measurement (Table 2) showed lower δ values for this compound. Interestingly, the peak of 5-androstene-3β,17α-diol was not increased in size nor did it show reduced δ value, implying that this compound is not a metabolite of ingested DHEA. 5-Androstene-3β,17α-diol may not even be a major metabolite of in vivo produced DHEA since Weusten et al.[16] suggest that it is biosynthesized as a byproduct of the direct conversion of

Table 2 $\delta^{13}C‰$ values for androstenediol and androstanediol components following steroid ingestion

Steroid	Baseline	DHT	epiT	DHEA
5β-Androstane-3α,17α-diol	−25.90	NM	−30.96	−27.67
5β-Androstane-3α,17β-diol	−26.48	−26.51	NM	−29.60
5α-Androstane-3α,17α-diol	NM	−30.53	−29.60	NM
5α-Androstane-3α,17β-diol	−25.64	−30.80	NM	−29.14
5-Androstene-3β,17α-diol	−24.33	−27.19	NM	−26.39
5-Androstene-3β,17β-diol	−25.86	−27.88	NM	−29.25
Pregnanetriol (ERC)	−25.85	−26.50	−26.43	−26.06

IRMS $\delta^{13}C‰$ values obtained by measurement of diol fractions obtained 6 h after administration of 50 mg of synthetic androgens. The values "boxed" represent those showing significant reduction which indicates that the compounds were largely metabolites of exogenous synthetic steroids. NM, not measured.

USADA 0757

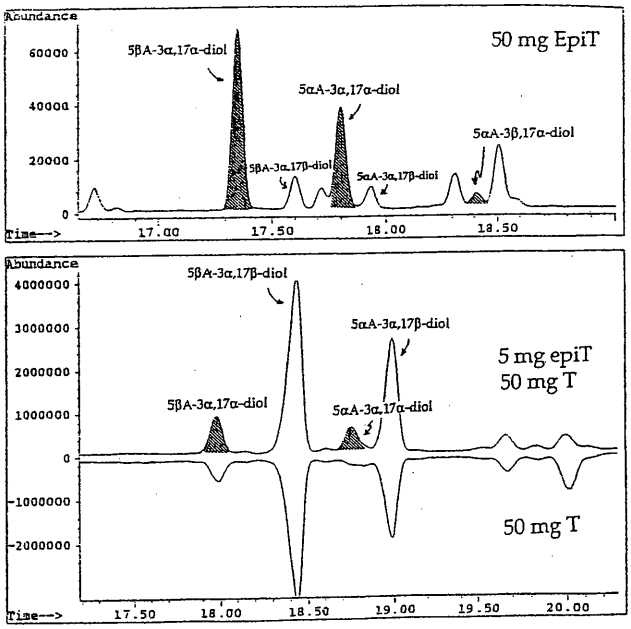


Figure 4 GC profiles following epiT administration. Only the central parts of the chromatograms are illustrated. The upper panel shows dominance of 17α-metabolites following a 50-mg epiT dose and these were shown to have significant reduction of $\delta^{13}C‰$ values. The experiment was repeated following 5 mg of epiT administration in conjunction with 50 mg of testosterone, providing a chromatogram (middle panel) more typical for actual use of epiT as a steroid taken to normalize the urinary T/epiT ratio. The lower ("mirror image") panel shows profile following testosterone administration alone.

pregnenolone to 5,16-androstanedien-3β-ol. Despite the prevailing notion that once oxidized, 17β-hydroxysteroids are not reduced to a significant extent,[17–19] 5α- (and 5β)-androstanediols did give lowered δ values.

Pregnenolone administration

Pregnenolone was primarily metabolized by 20α-hydroxysteroid dehydrogenase to 5-pregnene-3β,20α-diol. In contrast to the situation after DHEA administration, the peak in the chromatogram (Figure 5, bottom), representing 5-androstene-3β,17α-diol, when measured against its 17β-epimer, doubled in size. This would support the concept that the 17α-diol is a pregnenolone metabolite, but not one of 17α-hydroxypregnenolone or DHEA.[16] IRMS measurements were not made on the samples collected in the study.

Practicality of method and comparison to other studies

Optimum IRMS data require baseline separation of GC components so that (1) there is no secondary component that may alter the measured δ value and (2) integration can be accomplished over the whole peak to avoid a chromatographic isotope effect.[23] The requirements therefore differ greatly from GC/MS (in selected ion-monitoring mode) where 100% overlap of components is perfectly acceptable, so a major simplification of urinary steroid extracts is necessary before IRMS analysis to allow complete resolution of analytes. Another differing requirement pertains to derivatization. While in GC/MS trimethylsilyl ether (TMS) or methyloxime-TMS derivatives are those of choice because of their rich mass spectra, providing many ions suitable for selective ion monitoring, these derivatives are unsuitable for IRMS. It has been found that SiO_2 formed during combustion soon inactivates the oxidation catalyst. It would be preferable to avoid the use of derivatization altogether, but if more than one or two analytes are present in the GC chromatogram this is impossible because of increased peak width and tailing. While acetylation may appear to be undesirable because it could potentially contribute to isotope fractionation during derivative formation,[23] in practice this has not been shown to be significant in steroid analyses, so the acetates have become the derivative of choice.[2,3] Although any additional carbons are undesirable, acetylation only adds four to the diols whereas silylation would add six, a secondary reason for not using the latter reaction. Choice of GC chromatography column is also important. For GC/MS steroid analysis of TMS and MO-TMS derivatives, nonpolar OV-1 (methylsilicone) type columns (e.g., DB-1) are usually chosen because derivatized urinary steroids of increasing complexity are spaced almost equidistantly within a linearly programmed chromatogram. This is not the case for separation on polar or medium polarity columns where the polarity and stereochemistry of individual steroids plays a greater part in separation. It appears, however, that nonpolar columns give rise to greater isotope fraction-

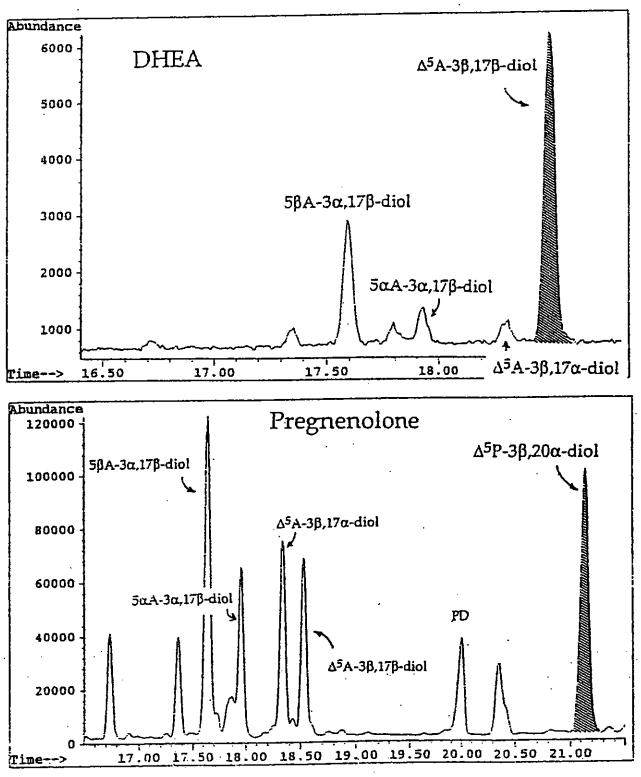


Figure 5 Diol profiles following the administration of the Δ^5 steroids DHEA and pregnenolone highlighting the appropriate analytes for IRMS measurement. Only central parts of chromatograms illustrated.

USADA 0758

ation between isotopomers than do polar or medium polarity columns.[23] This is an undesirable feature for IRMS and can lead to compromised results, particularly if peaks are not baseline separated or completely integrated from beginning to end. In our studies, there was no discernible separation between ^{12}C and ^{13}C peaks containing androstanediol, indicating suitability of the column chosen. It must be borne in mind that the greatest degree of isotope fractionation occurs for CO_2 and $^{13}CO_2$ between combustion chamber and mass spectrometer collector.

There have been three major approaches to use of the IRMS technique in natural steroid doping confirmation. In their pioneering study, Becchi et al.[2] utilized β-glucuronidase hydrolysis of urinary steroids with low resolution silica cartridge fractionation and preparative high performance liquid chromatography (HPLC) resolution of testosterone, DHEA, and androstanediol fractions. Urinary cholesterol, separately extracted, was used as internal standard (ERC). Acetylation was carried out preceding GC/IRMS. In an adapted method,[3] they carried out acetylation prior to HPLC analysis to improve purity of the isolated components but this change had the disadvantage that DHEA could not be separately isolated, although 5-androstene-3β,17β-diol could be resolved. This latter compound provided the "baseline" value for δ since it is a precursor of testosterone, not a catabolic product. Use of this ERC would not be possible if the method was used on an individual who had been taking DHEA in addition to testosterone since the androstanediol is a major metabolite. However, the fact that 5-androstene-3β,17β-diol was separately isolated means that the method, essentially unchanged, could be utilized for proving DHEA administration through $\delta^{13}C‰$ measurement of the androstenediol. In their studies 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol diacetates co-eluted, preventing separate δ value measurement, but these epimers could certainly be separated by choosing a polar GC column. Horning et al.[4] have also described a technique for IRMS of urinary testosterone and metabolites. Their methodology also involved β-glucuronidase hydrolysis, but a novel immunoaffinity column (with selectivity towards 17β-hydroxy steroids) was employed, simplifying the steroid extract by primarily retaining testosterone and the androstanediols. Testosterone, 5α- and 5β-androstane-3α,17β-diol fractions were individually collected following HPLC of the immunoaffinity column fraction and these were subjected to GC/IRMS analysis underivatized. Because the 5α- and 5β-androstanediols were separately determined, this method can be used to distinguish testosterone from DHT misuse (S. Horning, personal communication, 1997). Presumably, their method could also be used to extract and analyze another 17β-steroid, the DHEA metabolite 5-androstene-3β,17β-diol. However, as is also the case in Aguilera's method,[3] this steroid could be obtained only from the glucuronide fraction unless a different hydrolytic enzyme preparation was used. Sensitivity of detection may therefore be a problem since the androstenediol is predominantly excreted as mono- and disulfate conjugates, not as a glucuronide (Table 1). The androstanediol products of epiT metabolism could not be isolated by this method unless a separate 17α-immunoaffinity column was developed and utilized.

Our approach to the use of IRMS has been somewhat different in that we have endeavored to produce a single high resolution GC chromatogram separating all analytes required to definitively establish whether any of four exogenous androgens was being administered. Prior to the GC analysis, we have sought to keep procedures low resolution to minimize the possibility of isotope fractionation, curtail instrument costs and facilitate batch processing. To minimize GC isotope fractionation, we utilize a medium polarity DB-17 column,[24] which fortuitously also allows the separation of 5α- and 5β-androstane-3α,17β-diols, unlike DB-1-like phases. In our methodology the low resolution fractionations include 1) removal of the urinary ketonic steroids which amount to about 75% of the total, 2) selection of an extraction solvent which only removes steroids equal to and below the polarity of pregnanetriol (a further 15% steroids and sterols removed), and 3) chromatography mini-LH-20 columns (which removed about another 2% of original urinary steroids and a major part of nonsteroidal impurities). The final extract of diols and pregnanetriol contains about 8% in mass of the original urinary steroids. We elected to use *H. pomatia* hydrolysis for deconjugation since this improves recovery of 3-monosulfates of 5-androstene-3β,17β (and 17α)-diol and 5-pregnene-3β,20α-diol. A criticism in the use of *H. pomatia* in analyses associated with doping control is that the enzyme preparation contains 3β-hydroxysteroid dehydrogenase which may result in the production of testosterone and androstenedione from DHEA and 5-androstene-3β,17β-diol, respectively.[24,25] However, minimal oxidation of Δ^5 steroids occurs if the hydrolysis is undertaken for a short period at high temperature (55°C), and in addition, any oxidative products formed would not remain in the analyte fraction since they would be discarded as Girard hydrazones.

Overall, the data presented by different investigators are remarkably similar in spite of the different methodologies utilized. Typically, in each study $\delta^{13}C‰$ values for testosterone or metabolites which would be considered negative for misuse run around −25 to −27 and those positive −28 to −31. It would seem that a consensus on discriminant values could be readily achieved and such values be adopted as regulatory.

Influence of peak contamination

Regarding contamination of chromatographic peaks, it was previously stated that IRMS requires homogenous peaks and complete baseline separation for the technique to be useful. While this is highly desirable, some degree of a secondary component in a peak is tolerable; the question is how much? In Figure 6 we provide models of contamination that suggest that up to 20% of an extraneous (not originating from administered drug) component may be permissible while still giving drug-positive test results. In the inset to Figure 6, we have assumed that a hypothetical δ −30 "exogenous" analyte is contaminated with a δ −25 endogenous component. This component can be of four principal types: (1) the identical analyte, but of endogenous origin; (2) a co-eluting different analyte; (3) the tail of a different component; or (4) chemical noise often associated with extended solvent front or column bleed. We believe our

USADA 0759

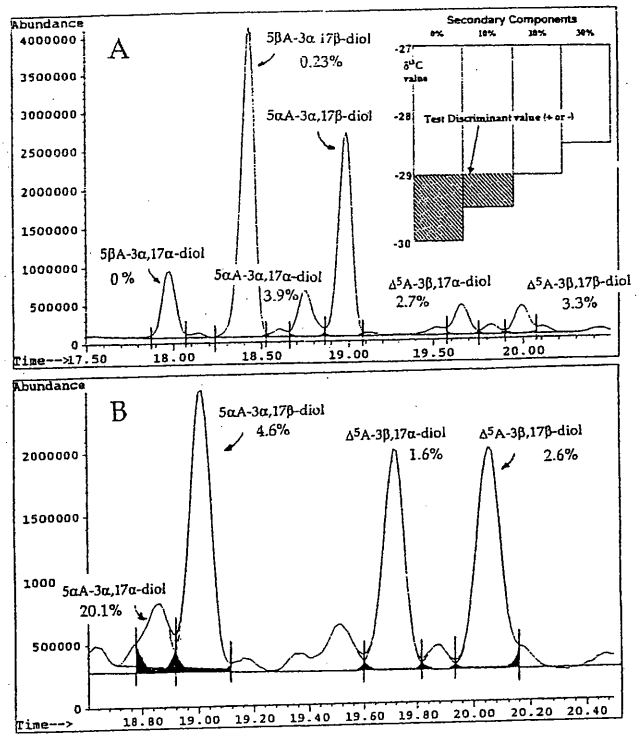


Figure 6 Influence of contamination of peak on major components. The inset illustrates how hypothetically contaminating a δ −30 with a δ −25 contaminant affects the overall δ value of a peak. At 20% contamination, the analyte value rises above a putative δ −29 value proposed for a positive drug test. Approximations are shown in chromatograms for negligible contamination (upper panel A, 50 mg T + 5 mg epiT administration) and more serious (lower panel B, no drug administration). The lower chromatogram had greater background remaining from "solvent front." The measured contaminant contributions are given as percentages on the chromatograms.

methodology excludes Item 2, and Item 1 must be accepted to some degree dependent on the amount of suppression of endogenous steroids by the administered hormone. Items 3 and 4 are real problems dependent on the quality of the samples and chromatographic separation. In Figure 6A we show an almost ideal chromatogram with the following strengths: it is from a drug-positive sample so ample steroid is present, reducing the relative amounts of chemical noise. Such a sample is also likely to have reduced amount of endogenous analyte because of hormone suppression. In this situation, contamination is limited to the minimal tails of adjacent peaks. In an example, we calculate peak contaminations from 0.23% for the major peak to 3.9% for 5α-androstane-3α,17α-diol, quite acceptable values within the context of the method.

Figure 6B illustrates a more compromised situation. It is from a control (no steroid drug) sample, but the lower quality chromatogram should not be considered typical for all control chromatograms. Quantitatively much less steroid is present in control samples so chemical noise and extraneous peaks contribute to a greater extent. The chromatographic baseline was sloping, indicating that nonspecific compound elution also contributed to the peak contents. In this case, we calculate extraneous material contributes between 1.6 and 20% for the different components. In reality, in this run only the result of the epitestosterone metabolite 5α-androstane-3α,17α-diol may be too compromised; the remaining components still show contaminations of <10%. Use of this androstanediol analyte is also not essential since the other epiT metabolite 5β-androstane-3α,17α-diol is always baseline separated. This chromatogram illustrates that the major contaminant may well be nonspecific solvent front components ("chemical noise") rather than tails of adjacent peaks. In doping control practice it may well be possible to detect δ values compromised because of this form of contamination by incorporating an internal standard of known δ value which is eluted before the elution of the androstanediol analytes. The current ERCs elute late in the chromatogram when all solvent front components have eluted. We propose using 5α-androstane-3β-ol as the internal standard (see Figure 2).

Conclusion

In summary, we have in this paper produced evidence that our testosterone metabolite methodology[5] could be improved and expanded to include metabolites of three other natural C_{19} steroids potentially misused in sport. The metabolites can be separated in a single chromatogram and ^{13}C could be individually measured by IRMS. For testosterone, proof of administration would be a lowered δ value for 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol; for DHT, proof would be lowered δ for 5α-androstane-3α,17β-diol and 5α-androstane-3α,17α-diol but not 5β-androstane-3α,17β-diol; and for epitestosterone, lowered δ for 5α-androstane-3α,17α-diol and 5β-androstane-3α,17α-diol. In the latter case, lowered ^{13}C content may also be expected for the 17β-epimers as anyone misusing epiT is also likely to be self-administering testosterone. DHEA administration would be proved by decreased $\delta^{13}C‰$ value for 5-androstene-3β,17β-diol. Each steroid drug has at least one metabolite separated to baseline so mutual contaminations need not be a concern.

We propose that the comprehensive nature of the methodology and relative simplicity of workup procedure should allow it to be a candidate technique for doping control application. With development it could be used for screening, possibly in place of the classical T/epiT and other diagnostic parameters. Clearly, this study is preliminary and merely illustrative of potential methodologies: extensive controlled studies with variable oral and injectable doses of the natural steroids will be necessary to prove the efficacy of the technique in regulating drug abuse in sports.

Finally, we have shown that the pregnenolone metabolite 5-pregnene-3β,20α-diol can also be analyzed using this technique. While not yet proscribed by sports organizations, pregnenolone is freely available from United States health food stores and may be banned in the future. Forbidding its use would presumably depend on whether its administration increases androgen production. Although pregnenolone synthesized in vivo is the precursor of all gonadal and adrenal steroids, its ingestion is unlikely to significantly raise androgen titers. In our single experiment, we did note that of the C_{19} steroids examined, only 5-androstene-3β,17α-diol showed a significant increase in GC peak size,

USADA 0760

a finding that would support evidence suggesting that the compound is a direct metabolite of pregnenolone (i.e., one formed not utilizing the accepted 17α-hydroxypregnenolone pathway). Clearly, detailed studies are necessary on the implications of regular pregnenolone administration to androgen production.

Note added at proof

Since acceptance of this manuscript, we have established that the unexpected DHT metabolite 5α-androstane-3α,17α-diol was essentially formed only after oral drug administration. Intramuscular (DHT heptanoate) and transdermal (Andractim® DHT cream) administration did not result in epimerization, 5α-androstane-3α,17β-diol being the only major nonketonic metabolite. The 17-epimerization occurring after oral anabolic steroid administration remains fascinating, and we are attempting to determine the site of this transformation.

References

1. Southan G, Mallet A, Jumeau J, Craig S, Poojara N, Mitchell D, Wheeler M, Brooks RV (1990). *Programme and Abstracts of the Second International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona. P. 306.*
2. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P (1994). Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rapid Commun Mass Spectrom* **8**:301–308.
3. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG Jr (1996). Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J Mass Spectrom* **31**:169–176.
4. Horning S, Geyer H, Machnik M, Schänzer W, Hilkert A, Oeßelmann J (1997). Detection of exogenous testosterone by $^{13}C/^{12}C$ analysis. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds), *Recent Advances in Doping Analysis*. SPORT und BUCH Strauß, Cologne, pp. 275–283.
5. Shackleton CHL, Phillips A, Chang T, Li Y (1997). Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediol. *Steroids* **62**:379–387.
6. Smith BN, Epstein S (1971). Two categories of $^{13}C/^{12}C$ ratios for higher plants. *Plant Physiol* **47**:380–384.
7. Catlin DH, Cowan DA, De la Torre R, Donike M, Fraisse D, Oftegro H, Hatton CK, Starcevic B, Becchi M, de la Torre X, Norli H, Geyer H, Walker CJ (1996). Urinary testosterone (T) to epitestosterone (E) ratios by GC/MS. I. Initial comparison of uncorrected T/E in six international laboratories. *J Mass Spectrom* **31**:397–402.
8. Donike M, Ueki M, Kuroda, Geyer H, Nolteernsting E, Rauth S, Schanzer W, Voekler E, Fujisaki M (1995). Detection of DHT doping: alterations in the steroid profile and reference ranges for DHT and its 5α metabolites. *J Sports Med Phys Fitness* **35**:235–250.
9. Baulieu EE, Mauvais-Jarvis P (1964). Studies on testosterone metabolism. I. Conversion of testosterone 17α-^{3}H to 5α- and 5β-androstane-3α,17β-diol,17α-^{3}H: A new '17β-hydroxylation pathway.' *J Biol Chem* **239**:1569–1577.
10. Schänzer W, Horning S, Donike M (1996). Synthesis and metabolism of [16,16,17-2H3]-5α-dihydrotestosterone. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds), *Recent Advances in Doping Analysis*. SPORT und BUCH Strauß, Cologne, pp. 201–213.
11. Shackleton CHL, Whitney JO (1980). Use of Sep-pak® cartridges for urinary steroids extraction: evaluation of the method for use prior to gas chromatographic analysis. *Clin Chim Acta* **107**:231–243.
12. Geyer H, Schänzer W, Schindler U, Donike M (1996). Changes of the urinary steroid profile after sublingual application of dihydrotestosterone (DHT). In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds), *Recent Advances in Doping Analysis*. SPORT und BUCH Strauß, Cologne, pp. 215–229.
13. Dehennin L, Matsumoto AM (1993). Long-term administration of testosterone enanthate to normal men: Alterations of the urinary profile of androgen metabolites potentially-useful for detection of testosterone misuse in sport. *J Steroid Biochem Mol Biol* **44**:179–189.
14. Dehennin L (1994). Detection of simultaneous self-administration of testosterone and epitestosterone in healthy men. *Clin Chem* **40**:106–109.
15. Coutts SB, Kicman AT, Cowan D (1996). Intramuscular administration of 5α-dihydrotestosterone heptanoate: Changes in the urinary hormone profile. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds). *Recent Advances in Doping Analysis*. SPORT und BUCH Strauß, Cologne, pp. 173–183.
16. Weusten JJAM, Legematt G, Van der Wouw MPME, Smales AGH, Kloppenborg PWC, Benraad TH (1989). The mechanism of the synthesis of 16-androstenes in human testicular homogenates. *J Steroid Biochem* **32**:689–694.
17. Dorfman RI, Ungar F (1965). *Metabolism of Steroid Hormones*. Academic Press, New York, pp. 289–381.
18. Massé R, Laliberte C, Tremblay L (1985). Gas chromatographic/mass spectrometric analysis of 19-nortestosterone: Urinary metabolites in man. *Biomed Mass Spectrom* **12**:115–121.
19. Gower DB, Houghton E, Kicman AT (1995). Anabolic steroids: Metabolism, doping and detection in equestrian and human sports. In: Makin HLJ, Gower DB, Kirk DN (eds), *Steroid Analysis*. Blackie Academic & Professional, London, pp. 468–526.
20. Macdonald BS, Sykes PJ, Adhikary PM, Harkness RA (1971). The identification of 17α-hydroxy-17-methyl-1,4-androstandien-3-one as a metabolite of the anabolic steroid drug 17β-hydroxy-17-methyl-1,4-androstadien-3-one in man. *Steroids* **18**:753–766.
21. Schänzer W, Opfermann G, Donike M (1992). 17-Epimerization of 17α-methyl anabolic steroids in humans: Metabolism and synthesis of 17α-hydroxy-17β-methyl steroids. *Steroids* **57**:537–550.
22. de Boer D, Gainza Bernal ME, van Oyen RD, Maes RAA (1991). The analysis of trenbolone and the human urinary metabolites of trenbolone acetate by gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry. *Biol Mass Spectrom* **20**:459–466.
23. Meier-Augenstein W (1997). The chromatographic side of isotope ratio mass spectrometry: Pitfalls and answers. *LC-GC International* (Jan):17–25.
24. Vanluchene E, Eechaute W, Vanderkerckhove D (1982). Conversion of free 3β-hydroxy-5-ene steroids by incubation with *Helix pomatia*. *J Steroid Biochem* **16**:701–703.
25. Messeri G, Cugneto G, Moneti Gea (1984). *Helix pomatia*-induced conversion of some 3β-hydroxysteroids. *J Steroid Biochem* **20**:793–796.

USADA 0761

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 13, 2237–2243 (1999)

Analysis of Exogenous Dehydroepiandrosterone Excretion in Urine by Gas Chromatography/ Combustion/Isotope Ratio Mass Spectrometry

Makoto Ueki* and Masato Okano

Doping Control Laboratory, Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., 3-30-1 Shimura, Itabashi-ku, Tokyo 174-8555, Japan

A detailed procedure for the analysis of exogenous dehydroepiandrosterone (DHEA) in urine by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) has been established for detecting doping with DHEA. The average δ-value (parts per thousand difference of $^{13}C/^{12}C$ ratio from the isotope ratio standard) of 26 synthetic steroids commercially available was -30.1 ± 2.6, and was significantly lower than that of human endogenous DHEA in urine of the world class athletes who had participated in the XVIIth Olympic Winter Games (-20.3 ± 2.1, n = 446). Although large inter-individual variations of urinary DHEA excretion were observed following a single oral administration of 50 mg of DHEA, no significant inter-individual difference was found when the excretion of exogenous DHEA was monitored in terms of δ-values using GC/C/IRMS; the minimum δ-values were observed around 6–8 h after the administration, and the values returned to the base level at over 72 h after the dosing. Thus, the deviations in δ-values of DHEA and its diol metabolites are considered to be conclusive evidence for detecting doping with DHEA. Some successful cases of detection of doping with DHEA from athletes are also reported. **Copyright © 1999 John Wiley & Sons, Ltd.**

Received 17 August 1999; Revised 20 September 1999; Accepted 21 September 1999

Dehydroepiandrosterone (DHEA) is a major androgenic steroid secreted from the adrenal gland.[1] It circulates in the blood stream in the free or the sulfated form, and undergoes a strong age-related decrease.[2,3] It is also a precursor of both male and female hormone steroids. Dietary supplements that contain DHEA or other testosterone precursors are commercially available in the US without a medical doctor's prescription. In sports societies, DHEA and other testosterone precursors have become a serious problem, because some athletes are abusing them not only for enhancing performance, but also for successfully cheating the steroid tests. Therefore, the Medical Commission of the International Olympic Committee (IOC) classified DHEA into the category of anabolic steroids and related compounds in 1997. The major basis for detecting the abuse of a natural steroid is its absolute or relative concentration. The judgement is made statistically by following its concentration at reference intervals.

The recent development of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) has allowed high-precision measurements of isotopic composition in each compound.[4] The main source of urinary human steroids is food cholesterol contained in beef, pork, poultry and fish, while most synthetic steroids are chemically derived from C3-plant products like soybean phytosterols. Thus, the carbon isotope ratio (CIR) of each steroid is identical to that of its starting material; the value does not depend on the urinary concentration, but is dependent on the ratio between an exogenous and an endogenous target compound. Previous reports on steroid research by GC/C/IRMS have mainly focused on testosterone[5–7] and some other non-intermediate steroid hormones;[8,9] little information is available for GC/C/IRMS of exogenous DHEA, despite the recent prevalence of DHEA-containing dietary supplements. In this paper, we describe a detailed procedure for detection of doping with DHEA and other naturally occurring steroids by means of GC/C/IRMS.

*Correspondence to: M. Ueki, Doping Control Laboratory, Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., 3-30-1 Shimura, Itabashi-ku, Tokyo 174-8555, Japan.
E-mail: wd3m-uek@asahi-net.or.jp

EXPERIMENTAL

Chemicals

DHEA, 4-androstene-3,17-dione, testosterone, epitestosterone, epiandrosterone, 5 α-androstane-3α, 17β-diol, 5β-androstane-3α, 17β-diol, 5α-androstane-3β, 17β-diol and 5α-androstan-3β-ol (internal standard) were obtained either from Sigma (St. Louis, MO, USA) or Steraloids Inc. (Newport, RI, USA). Androstat-100 (DHEA 100mg/tablet), Schiff-DHEA (DHEA 25mg/tablet), New-DHEA (DHEA 25mg/tablet), Mylis (injectable DHEA sulfate 100mg/ample) and DHEA acetate, were obtained from Bodyonics (Hicksville, NY, USA), Schiff Products (Salt Lake City, UT, USA), Leiner Health Products Inc. (Carson, CA, USA), Kanebo Co., Ltd (Tokyo, Japan) and Wako Pure Chemicals (Osaka, Japan), respectively. Other steroid products and their esters were also obtained from Japanese commercial sources as listed in Table 1, and were checked by GC/MS before use. Glufatase type A-II (β-glucuronidase:arylsulfatase = 42,000 Fishman U:21,000 Roy U per mL each) from *Ampullaria* was purchased from Nippon Bio-Test (Tokyo, Japan); β-glucuronidase from *E. coli* (80 unit/mL) from Boehringer Mannheim (Düsseldorf, Germany); and NBS19-limestone, an internationally certified reference material for CIR calibration, from NIST (Gaithersburg, MD, USA). All

CCC 0951–4198/99/222237–07 $17.50

Copyright © 1999 John Wiley & Sons, Ltd.

organic solvents were of analytical grade, HPLC grade or pesticide analysis grade. Sephadex LH 20 and Bond Elut C_{18} LRC 10cc/500mg were products of Pharmacia AB (Uppsala, Sweden) and Varian (Walnut Creek, CA, USA), respectively. Lipophylic anion exchange gel, triethylaminohydroxypropyl Sephadex LH 20 (TEAP-LH 20), was prepared according to the procedure of Axelson *et al.*[10]

Urine samples

Two tablets of 25 mg DHEA were self-administered orally by five healthy male volunteers. They were informed in advance of the details of the human experiments, and their consent was obtained. Baseline urine samples were obtained before administration, and the subsequent random urine samples were collected separately for the first 72 h after administration. Real urine samples were collected from athletes at in- and out-of-competition tests on a routine basis. Reference urine samples (24-h pool) were collected from 10 healthy female and 10 healthy male Japanese subjects. Urine samples from about 450 athletes who had participated in the XVII Olympic Winter Games, Nagano, 1998, were also analyzed.

Urinary steroid profiles were examined for all samples according to our GC/MS procedure published in the literature.[11,12] Urinary concentrations of testosterone, epitestosterone, DHEA, 5α- and 5β-androstane-3α,17β-diols, androsterone, etiocholanolone, 5α-dihydrotestosterone (DHT), and the ratios of the 5α-steroids to the corresponding 5β-steroids, were measured.

Sample preparation

The method started with fractionation of urinary steroids into free, glucuronyl- and sulfo-conjugated neutral steriods, enzymatic hydrolysis of the conjugates, and the subsequent formation of acetates of the steroid aglycone.

After centrifugation of a urine sample, 10–20 mL of the aliquot were applied onto a Bond Elut C_{18} cartridge and washed with the same volume of distilled water. The steroid fraction was eluted with 4 mL of methanol, and 1.5 mL of distilled water were added to the eluate. Then the eluate was applied onto a TEAP-LH20 anion exchange column (1.25 mL wet volume). The free, glucuronyl- and sulfo-conjugated steroids were eluted with 4 mL of 72% methanol, 8 mL of 0.4 M formic acid in 72% methanol and 8 mL of 0.5 M acetate in 72% methanol, pH = 10.0, respectively. Enzymatic hydrolysis of sulfates of 3β-hydroxy steroids was done by incubation with 30 µL of glufatase at 60 °C for 60 min, and that of glucuronides was done with 25 µL of β-glucuronidase from *E. coli* at 50 °C for 60 min. Ketoic steroids in the glucuronide fraction were then removed by the formation of hydrazones using (carboxymethyl)trimethylammonium chloride hydrazide according to a procedure similar to that reported by Shackleton *et al.*[8] A 24 µL aliquot of internal standard solution (2.5 µg of 5α-androstan-3β-ol) was added to the steroid aglycones and they were extracted with 5 mL of *n*-pentane. The dried residue of the extract was dissolved in 100 µL of dry pyridine. The steroid acetate was formed by heating with 200 µL of acetic anhydride at 60 °C for 60 min. The excess derivatizing reagent was removed under a nitrogen stream, and the residue was re-dissolved in 30 µL of cyclohexane.

GC/C/IRMS

An HP6890 series GC instrument (Hewlett Packard, Palo Alto, CA, USA) was equipped with a 50%-phenylmethylsilicone DB-17 capillary column. (0.25 mm i.d. × 30 m, 0.25 µm film thickness) and 2µL of the final extract were injected in the splitless mode using a 6890 auto-sampler. The injection temperature was 260 °C, and the oven temperature started at 50 °C (initial hold 1 min) and was increased at 20 °C/min to 250 °C, and then at 5 °C/min to 300 °C (final hold 9 min). The carrier gas was helium at a flow rate of 1 mL/min. The outlet of the GC column was connected to a micro glass column packed with copper oxide pellets placed in a combustion furnace maintained at 850 °C. The combustion gases were passed through a cryo-trap for removal of water, and the abundance ratios between the dried $^{13}CO_2$ and $^{12}CO_2$ were analyzed on an Isoprime isotope ratio mass spectrometer (Micromass, Manchester, UK). An electron impact ion source was used with an ionization current of 400 µA, and the ions at *m/z* 44, 45 and 46 were monitored by three independent Faraday ion collectors. The δ-value of carbon atoms is defined as parts per thousand deviation of isotopic compositions from that of Vienna PeeDee Belenite (PDB), and is calculated according to the following equation:

$$CIR = {}^{13}CO_2/{}^{12}CO_2$$

$$\delta^{13}C_{Sample} = [(CIR_{Sample} - CIR_{PDB})/CIR_{PDB}] \times 1,000(‰)$$

For daily analyses, a standard carbon dioxide gas, that had been calibrated against the certified reference carbon dioxide obtained from NBS19-limestone by reaction with 85% H_3PO_4, was used.[13] Negative shifts of the δ-value of a sterol due to the formation of an acetate was corrected as follows:[14]

$$D_{OH} = D_{OAC} + 2m(D_{OAC} - D_{AC})/n$$

where

D_{OH}: $\delta^{13}C$ for the underivatized steroids
D_{OAC}: $\delta^{13}C$ for the acetylated steroids
D_{AC}: $\delta^{13}C$ for the acetylating reagent
n: number of carbon atom in a molecule
(for androstanes: n = 19)
m: number of hydroxyl groups to be acetylated
(for DHEA: m = 1)

RESULTS AND DISCUSSION

Measurements of δ-values of commercially available synthetic steroids

Commercially available steroid standards, or active ingredients of pharmaceutical preparations, were analyzed for their δ-values by GC/C/IRMS without any chemical transformation (Table 1). The average δ-value of 26 different synthetic steroid products was −30.1 with a standard deviation of 2.6. The value agreed well with that of C3-plant products,[15] and the value was lower than that for urinary human steroids. The average δ-values for free steroids and steroid esters were −30.2 and −29.9, respectively, and no significant difference was found between them.

Copyright © 1999 John Wiley & Sons, Ltd.

USADA 0763

Table 1. δ-Values calculated for synthetic steroids

Product name	Generic name	Manufacturer	δ-Value
Androstenedione	4-Androstene-3, 17-dione	Sigma Chemical Co.	−28.7
Androstenedione	4-Androstene-3, 17-dione	Tokyo Kasei Kogyo Co., Ltd.	−26.1
Stanolone	5α-Dihydrotestosterone	Tokyo Kasei Kogyo Co., Ltd.	−28.1
Dehydroepiandrosterone	DHEA	Tokyo Kasei Kogyo Co., Ltd.	−31.4
Dehydroisoandrosterone	DHEA	Sigma Chemical Co.	−33.5
Dehydroisoandrosterone	DHEA	Wako Pure Chemical Industries., Ltd.	−34.1
New DHEA	DHEA	Leiner Health Products Inc.	−30.5
Schiff DHEA	DHEA	Schiff Products	−30.6
17α-Epitestosterone	Epitestosterone	Sigma Chemical Co.	−33.1
BHP5	Pregnenolone	Bodyonics	−31.2
Pregnenolone	Pregnenolone	Tokyo Kasei Kogyo Co., Ltd.	−30.5
Testosterone	Testosterone	Sigma Chemical Co.	−28.4
Testosterone	Testosterone	Tokyo Kasei Kogyo Co., Ltd.	−28.4
Testosterone	Testosterone	Wako Pure Chemical Industries Ltd.	−28.2
Dehydroepiandrosterone Acetate	DHEA acetate	Tokyo Kasei Kogyo Co., Ltd.	−35.6
Dehydroisoandrosterone Acetate	DHEA acetate	Wako Pure Chemical Industries., Ltd.	−34.5
Mylis	DHEA sulfate	Kanebo Co., Ltd.	−27.1
Testosterone Acetate	Testosterone acetate	Sigma Chemical Co.	−32.8
Enarmon Deot	Testosterone enanthate	Teikoku Zoki Pharmaceutical Co., Ltd.	−26.2
Testinon Depot 125mg	Testosterone enanthate	Mochida Pharmaceutical Co., Ltd.	−26.8
Testoviron-Depot	Testosterone enanthate	Schering AG	−27.4
TESTRON DEPOT-S	Testosterone enanthate	Fuji Pharmaceutical Co., Ltd.	−29.9
Enarmon	Testosterone propionate	Teikoku Zoki Pharmaceutical Co., Ltd.	−30.1
Testinon 10mg	Testosterone propionate	Mochida Pharmaceutical Co., Ltd.	−29.6
Testosterone Propionate	Testosterone propionate	Tokyo Kasei Kogyo Co., Ltd.	−30.1
Testosterone Propionate	Testosterone propionate	Wako Pure Chemical Industries., Ltd.	−28.9

Reference urine samples from Japanese subjects

Several key steroids for detection of natural hormone doping were measured for 10 healthy female and 10 healthy male Japanese subjects. In all cases, 5α- and 5β-androstane-3α, 17β-diols and pregnanediol (P2) were the major diol steroids of the glucuronide fraction. In the sulfate fraction, DHEA was usually observed as the most intense peak; however, there was great inter-individual variations in the DHEA concentrations. Typical GC/C/IRMS chromato-

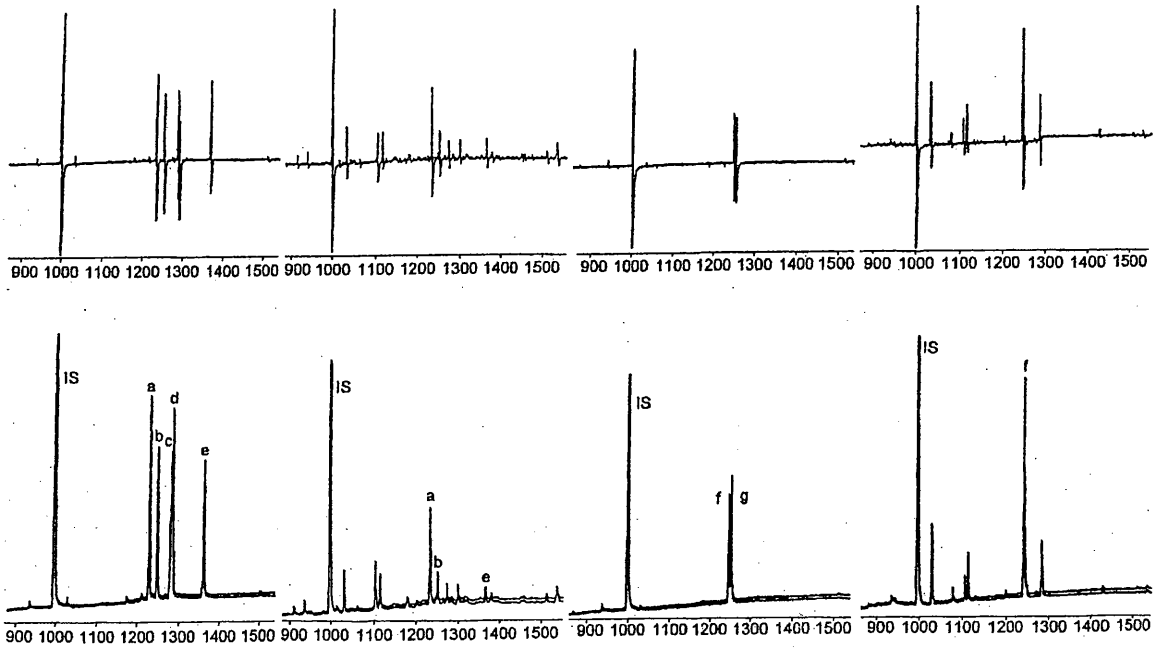


Figure 1. Typical GC/C/IRMS chromatograms of urinary steroids of a normal subject. Top trace shows the abundance ratios of ions *m/z* 45/44, and the bottom trace shows the normalized mass chromatograms of ions *m/z* 44 and 45. X-axis; retention time (sec); From left (a) Standard for glucuronide fraction 300 ng each, (b) Urine extract of glucuronide fraction, (c) Standard for sulfate fraction 300 ng each, (d) Urine extract for sulfate fraction. Peaks identified by letters; a,5β-diol; b,5α-diol; c,5-androstane-3α,17β-diol; d,5α-androstane-3β,17β-diol; e,P2; f,DHEA; g,epiandrosterone; IS, internal standard; Abbreviations, see Table 4.

Copyright © 1999 John Wiley & Sons, Ltd.

USADA 0764

Table 2. Absolute δ-values of DHEA and the diol metabolites, and their ratios to that for pregnanediol obtained from 20 normal Japanese volunteers

	Sex	n	Mean	SDn-1	Range
Absolute δ-values					
DHEA	M	10	−19.0	0.9	−17.2 to −20.8
	F	10	−18.6	1.0	−16.6 to −20.6
5 α-Androstane-3 α, 17 β-diol	M	10	−16.4	2.7	−11.0 to −21.8
	F	10	−15.5	2.4	−10.7 to −20.3
5 β-Androstane-3 α, 17 β-diol	M	10	−19.0	1.8	−15.4 to −22.6
	F	10	−17.5	2.7	−12.1 to −22.9
Pregnanediol (P2)	M	10	−20.6	0.9	−18.8 to −22.4
	F	10	−20.0	0.5	−19.0 to −21.0
Ratio of δ -values (/P2)					
DHEA/P2	M	10	0.92	0.03	0.86 to 0.98
	F	10	0.93	0.04	0.85 to 1.01
5 α-Androstane-3 α, 17 β-diol/P2	M	10	0.79	0.13	0.53 to 1.05
	F	10	0.78	0.12	0.54 to 1.02
5 β-Androstane-3 α, 17 β-diol/P2	M	10	0.92	0.08	0.76 to 1.08
	F	10	0.88	0.12	0.64 to 1.12

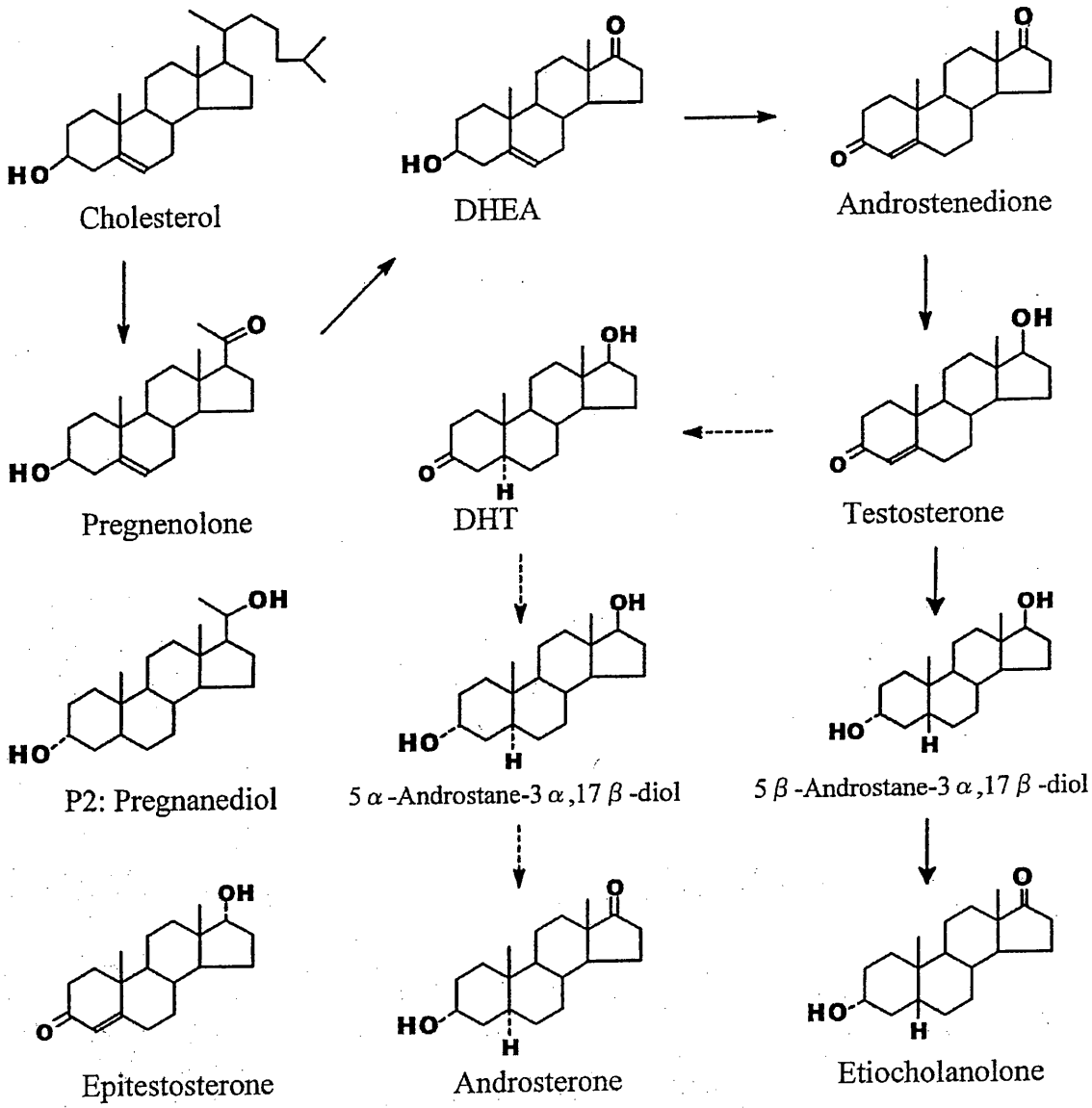


Figure 2. Metabolic pathways of androgens.

Copyright © 1999 John Wiley & Sons, Ltd.

USADA 0765

Table 3. Absolute δ-values of DHEA and the diol metabolites, and their ratios to that for pregnanediol obtained from Nagano Winter Olympic athletes (non-doping suspects)

	n	Mean	SDn-1	Range
Absolute δ-values				
DHEA	445	−20.3	2.13	−15.6 to −24.1
5 α-Androstane-3 α, 17 β-diol	352	−17.5	3.53	−10.5 to −24.6
5 β-Androstane-3 α, 17 β-diol	435	−20.0	2.75	−15.2 to −26.2
Pregnanediol (P2)	444	−21.0	1.65	−17.2 to −23.8
Ratio of δ-values (/P2)				
DHEA/P2	375	0.97	0.367	0.77 to 1.13
5 α-Androstane-3 α, 17 β-diol/P2	349	0.86	0.163	0.47 to 1.12
5 β-Androstane-3 α, 17 β-diol/P2	433	0.96	0.098	0.73 to 1.12

grams of urinary diol steroids in the glucuronide fraction, and of DHEA in the sulfate fraction, obtained from a normal subject, are shown in Fig. 1. The δ-values of the four steroids obtained from 20 normal subjects are listed in Table 2. The δ-values were evaluated by a Smirnov-Grubbs calculation[16] and no outlying data were found by the rejection test. In all steroids, the mean δ-value was about −19 and they were distributed in the range of −10 to −23. No human steroid showed a lower δ-value than those for the synthetic steroids listed in Table 1, and no sex difference was found (Table 2). The ratios of δ-values for DHEA and androstanediols to that for P2 are also listed in Table 2. For all three steroids, the mean ratios of the δ-values were lower than 1.00, and their ranges were between 0.85 and 1.12. In a previous report,[9] the δ-value of P2 was used as a correction factor for diol steroids. Because conversion of DHEA to P2 does not take place (Fig. 2), the δ-value of P2 is not influenced by the administration of DHEA. Therefore, the δ-value of P2 in a urine sample of each individual can also be used for DHEA as a good reference to correct for the variation of the δ-values affected by individual food customs or by ethnic food differences.

Reference urine samples from the Olympic Winter Games in Nagano

Absolute δ-values of three target steroids (DHEA, 5α- and 5β-androstane-3α, 17β-diols), and their ratios to that of P2, were measured on about 450 athletes who had participated in the XVII Olympic Winter Games in Nagano, Japan. Urine samples which had shown any trace of drug use were not involved in this population. The results were evaluated by a Smirnov-Grubbs calculation[16] as above, and one or two outlying data per compound were rejected. The calculated reference data are listed in Table 3. No information to identify the athletes was given to our laboratories by the Medical Commission of the IOC, but a majority of the subjects were Caucasians. The mean δ-values (Table 3) were 1 to 2 units more negative than those for the Japanese subjects (Table 2), but these differences were not significant. Further, the ratios to P2 fell within the same range as those for the Japanese. The mean δ-value for 5α-androstane-3α, 17β-diol is about 2 units higher than those for 5β-androstane-3α, 17β-diol, and this was also the case in normal Japanese subjects (Table 2). No significant sex difference was found in this population either. Thus, the method proved to be applicable to subjects of either Mongoloid or Caucasian ethnicity.

Time courses of urinary DHEA and δ-values after oral administration of DHEA

Oral administration of 50 mg of DHEA resulted in an increase of urinary DHEA excretion and a negative shift in the δ-values of DHEA, but the profiles of DHEA concentration suffered from large inter-individual differences (Fig. 3). In all subjects, the δ-values of DHEA

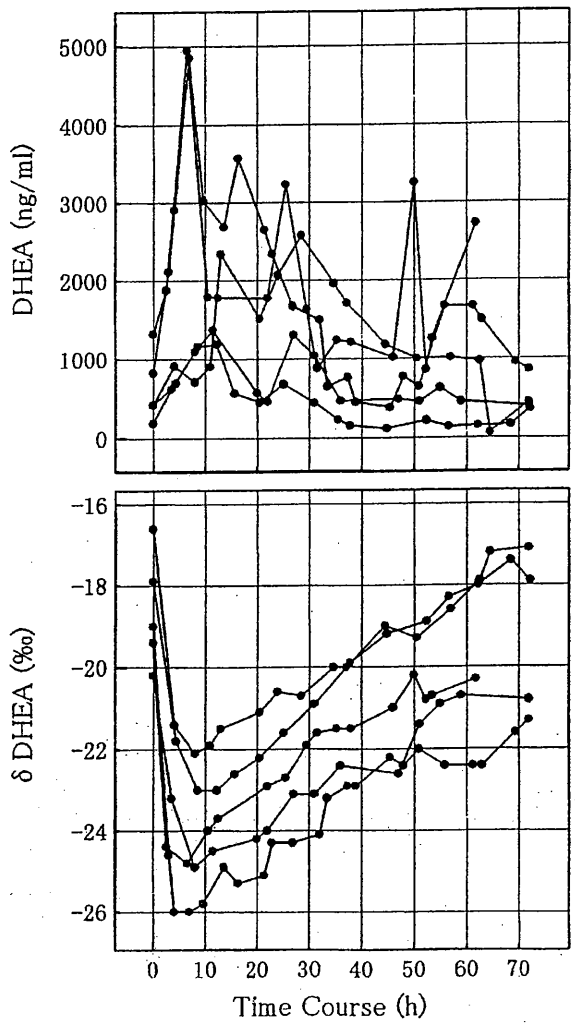


Figure 3. Time courses of urinary DHEA concentrations and δ-values after oral administrations of 50 mg of DHEA.

Copyright © 1999 John Wiley & Sons, Ltd.

USADA 0766

Table 4. Steroid profiles of some athletes suspected of doping with naturally occurring steroids

Sample No.	Sex	Concentration (ng/mL)								Absolute δ-value (‰)				Ratio of δ-value (/P2)		
		T	ET	A	E	DHT	5 α-diol	5 β-diol	DHEA	DHEA	5 α-diol	5 β-diol	P2	DHEA	5 α-diol	5 β-diol
1	M	1210	26	1250	6060	36	343	1490	38	−19.1	−27.0	−27.8	−20.0	0.96	1.35	1.39
2	M	4	19	5400	2110	301	825	67	1500	−18.2	−27.3	−17.5	−18.3	0.99	1.49	0.96
3	M	14	107	10000	9930	16	292	369	736	−16.7	−27.1	−28.5	−19.5	0.86	1.39	1.46
4	M	11	74	2100	12000	8	137	449	493	−14.7	−30.0	−29.5	−18.0	0.82	1.67	1.64
5	M	4	14	4550	3740	4	57	51	157	−21.2	−29.0	−28.6	−19.1	1.11	1.52	1.50
6	M	4	19	5400	5730	3	119	169	1593	−23.9	−24.0	−25.1	−17.5	1.37	1.37	1.43
7	M	29	21	2000	3750	6	34	146	2410	−25.4	−23.8	−22.7	−14.6	1.74	1.63	1.55
8	M	64	48	7180	24100	51	150	1730	2470	−30.3	−26.7	−28.6	−15.7	1.93	1.70	1.82
9	M	5	17	7520	8850	1	258	359	4030	−25.0	−29.2	−29.1	−19.2	1.30	1.52	1.52
10	F	2	27	1720	1890	1	21	30	4660	−17.0	−19.7	−22.1	−21.1	0.81	0.93	1.05

Abbreviations T: Testosterone, ET: Epitestosterone, A: Androsterone, E: Etiocholanolone, DHT: Dihydrotestosterone, P2: Pregnanediol 5 α-diol: 5 α-androstane-3 α, 17 β-diol, 5 β-diol: 5 β-androstane-3 α, 17 β-diol, DHEA: Dehydroepiandrosterone.

decreased by 4 to 6 (‰) units within the first 8 h, and returned to the base levels gradually over 72 h without exception, showing the usefulness of the δ-values for detection of doping with DHEA. After the intake of DHEA, the absolute δ-value of DHEA and ratios to P2 for DHEA, 5α-androstane-3α, 17β-diol and 5β-androstane-3α, 17β-diol moved significantly (data not shown), in accordance with the metabolic pathways (Fig. 2). Similarly, in all excretion studies, no significant changes in the δ-values of P2 were observed. Thus, the above results show that the ^{13}C-contents of steroids in terms of δ-values provide an excellent parameter to detect doping with synthetic steroids.

Case studies

The present GC/C/IRMS procedure has been applied to routine doping tests in our laboratories since 1997. Table 4 shows some actual examples of our tests for athletes who had been suspected for doping with naturally occurring steroids. Nos. 1 and 2 were testosterone- and DHT-positive, according to the conventional criteria based on the steroid profiles. An elevated ratio of testosterone to epitestosterone was evident in No. 1, and the high DHT concentration and elevated ratios of 5α- to 5β- hydroxy steroids supported doping with DHT in No.2.[11] The negative shift of the δ-value of 5β-androstane-3α, 17β-diol after DHT administration was negligible because inter-conversion between 5α- and 5β-androstanediols does not occur, as reported in our previous paper.[11,17] Nos. 3–5 were urine samples collected from athletes who had admitted the administration of androstenedione. For athlete No. 6, information concerning recent medication was available, and administration of DHEA was evident.

Athletes 7–10 showed high urinary DHEA concentrations of 2410, 7470, 4030 and 4660 ng/mL, respectively. Elevated androsterone and etiocholanolone were also noted in athletes 8 and 9. The absolute δ-value of DHEA, and ratios of the δ-values of DHEA, 5α- and 5β-androstane-3α, 17β-diols to that of P2 in athletes 7–9, significantly deviated from the reference range (Tables 2–4), and the results were thus judged to be due to doping with DHEA. In contrast, these δ-parameters were not abnormal for No. 10; no signs of doping were detected in spite of her high urinary DHEA level, showing that exogenous and endogenous DHEA are successfully discriminated by our GC/C/IRMS method.

CONCLUSIONS

The major difficulties in the detection of DHEA doping are caused by large ethnic, age-dependent and inter-individual variations of urinary DHEA concentrations. Non-doping subjects with high DHEA excretion levels are more common than those with naturally elevated testosterone. Therefore, the frequency of doping with DHEA or other testosterone precursors is currently increasing. Previous studies by GC/C/IRMS were mainly focused on the detection of testosterone doping, and gave some useful results.[5–7] Because DHEA is a precursor for testosterone, additional parameters have to be monitored to discriminate between DHEA and testosterone dopings. Our method consists of the fractionation of steroid conjugates into free, glucuronyl and sulfated fractions, and measurements of δ-values of DHEA by GC/C/IRMS. The carbon isotope ratio does not depend on the concentration, but is definitively dependent on different sources of a target compound. In spite of the large inter-individual differences in the urinary DHEA concentrations (Fig. 3), suitably small variations in the δ-values of DHEA and their ratios to that of pregnanediol were found for all human ethnicities (Tables 2 and 3), thus permitting the potent discriminating power of GC/C/IRMS for detecting doping with naturally occurring steroids, including DHEA and other testosterone-related steroids.

REFERENCES

1. Baulieu EE, Corpèchot C, Dray F, Emiliozzi R, Lebeau MC, Mauvais-Jrvis P, Robel P. *Recent Prog. Horm. Res.* 1965; **21**: 411.
2. Dean W, Morgenthaler J, Fowkes SW. *Steroids and Aging: Smart Drugs II*, Health Freedom Publications: Petaluma 1997; 81.
3. Yamaji T, Ishibashi M, Takaku F, Teramoto A, Takakura K, Takami M, Fukushima T, Kamoi K. *Acta Endocrinol.* 1989; **120**: 655.
4. Sifer JA, Engel MH, Macko SA, Jumeau EJ. *Anal. Chem.* 1991; **63**: 370.
5. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. *Rapid Commun. Mass Spectrom.* 1994; **8**: 30.
6. Aguilera R, Becchi M, Casabianca H, Hatton C, Catlin DH, Starcevic B, Pope HG Jr. *J. Mass Spectrom.* 1996; **31**: 169.
7. Horning S, Geyer H, Machnik M, Schänzer W. In *Recent Advances in Doping Analysis 4*, Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds). Sport und Buch Strauß, Edition Sport: Cologne, Germany, 1997; 115.
8. Shackleton CH, Roitman E, Phillips A, Chang T. *Steroids* 1997; **62**: 379.

Copyright © 1999 John Wiley & Sons, Ltd.

USADA 0767

9. Shackleton CH, Roitman E, Phillips A, Chang T. *Steroids* 1997; **62**: 665.
10. Axelson M, Sahlberg B-L, Sjövall J. *J. Chromatogr.* 1981; **224**: 355.
11. Donike M, Ueki M, Kuroda Y, Geyer H, Norteernsting E, Rauth S, Schänzer W, Schindler U, Völker E, Fujisaki M. *J. Sports Med. Phys. Fit.* 1995; **35**: 1.
12. Ueki M. In *Biological Mass Spectrometry: Present and Future*, Matsuo T, Caprioli RM, Gross ML, Seyama Y (eds). John Wiley: Chichester, 1994; 631.
13. Friedman I, O'Neil JR, Cebula G. *Geostandards Newsletter*, 1982; **6**: 11.
14. Johnes DM, Carter JF, Eglinton G, Jumeau EJ, Fenwick CS. *Biol. Mass. Spec.*, 1991; **20**: 641.
15. Yuexia L, Wendy SW, Liang Y, Robert ES. *J. Chromatogr. A* 1998; **800**: 51.
16. Smirnov NV. *Ann. Math. Stat.* 1948; **19**: 279.
17. Ueki M, Okano M. *J. Toxicol. Toxin Rev.* 1999; **18**: 177.

Copyright © 1999 John Wiley & Sons, Ltd.

USADA 0768

ELSEVIER

Journal of Chromatography B, 727 (1999) 95–105

JOURNAL OF CHROMATOGRAPHY B

Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols

Rodrigo Aguilera[a,*], Don H. Catlin[a,b], Michel Becchi[c], Andy Phillips[d], Cristina Wang[e], Ronald S. Swerdloff[e], Harrison G. Pope[f], Caroline K. Hatton[a]

[a]*UCLA Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology, University of California at Los Angeles, 2122 Granville Avenue, Los Angeles, CA 90025-6106, USA*

[b]*UCLA Olympic Analytical Laboratory, Department of Medicine, University of California at Los Angeles, 2122 Granville Avenue, Los Angeles, CA 90025-6106, USA*

[c]*Service Central d'Analyse, CNRS, BP 22, 69390 Vernaison, France*

[d]*Micromass, Wythenshawe, Manchester, UK*

[e]*Division of Endocrinology, Department of Medicine, Harbor-UCLA Medical Center, Torrance, CA 90502, USA*

[f]*Harvard Medical School, McLean Hospital, 115 Mill Street, Belmont, MA 02178-9106, USA*

Received 1 November 1998; received in revised form 18 January 1999; accepted 4 February 1999

Abstract

We propose a new screening method for testosterone (T) doping in sport. The current method for detecting T administration is based on finding a T to epitestosterone ratio (T/E) in urine that exceeds six. The difficulties with T/E are that T administration does not always result in a T/E>6 and that a rare individual will have T/E>6 in the absence of T administration. Our previous studies reveal that carbon isotope ratio helps to determine the origin of the urinary T because the values for T and its metabolites decrease after the administration of exogenous T. In this study, we present a rapid and efficient screening sample preparation method based on three successive liquid–solid extractions, deconjugation with *E. coli* β-glucuronidase after the first extraction, acetylation after the second extraction, and a final extraction of the acetates. The $^{13}C/^{12}C$ of two T metabolites (5β-androstane-3α,17β-diol and 5α-androstane-3α,17β-diol) and one pregnanediol as endogenous reference (5β-pregnane-3α,20α-diol) was measured by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) on 10 ml of urine collected from 10 healthy men before and after T administration. Following T administration, the $^{13}C/^{12}C$ of 5β-androstane-3α,17β-diol diacetate and 5α-androstane-3α,17β-diol diacetate declined significantly from −26.2‰ to −30.8‰ and from −25.2‰ to −29.9‰, respectively and the $^{13}C/^{12}C$ of 5β-pregnane-3α,20α-diol diacetate was unchanged. In addition, the ratio of androstanediols to pregnanediol increased in the post-T urines.
© 1999 Elsevier Science B.V. All rights reserved.

Keywords: Steroids; Testosterone

1. Introduction

After 1976, when the use of anabolic androgenic steroids (AAS) was prohibited by the International Olympic Committee (IOC), several techniques were

*Corresponding author. Tel.: +1-310-825-2635; fax: +1-310-206-9077.
E-mail address: rodrigoa@ucla.edu (R. Aguilera)

0378-4347/99/$ – see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(99)00066-3

developed to detect these compounds. At present, AAS are detected in urine samples by gas chromatography–mass spectrometry (GC–MS). However, pharmaceutical or exogenous testosterone presents special challenges as it is not possible to distinguish it from natural endogenous testosterone by GC–MS.

New possibilities for detecting exogenous T emerged following reports that the carbon isotope ratio ($^{13}C/^{12}C$) of exogenous T was different from that of endogenous T [1]. The $^{13}C/^{12}C$ of natural human testosterone is a function of the aggregate sum of the $^{13}C/^{12}C$ of the plants and animals we consume, and any additional effects of human biological processing. Synthetic testosterone is prepared from one plant source with an unusually low $^{13}C/^{12}C$. These slight differences can be detected through isotope ratio mass spectrometry [2–6]. In a T administration study that collected urines from subjects before and after T administration, we reported that the $^{13}C/^{12}C$ of urinary T and metabolites of T were significantly lower after T, and that the $^{13}C/^{12}C$ of metabolic precursors of T were unchanged [2]. Shackleton et al. [5] confirmed that T administration results in lower $^{13}C/^{12}C$ values for T metabolites, showed that the values remain low for 8 days following a dose of 250 mg of T enanthate, and demonstrated the potential applicability of the method for detecting administration of dehydroepiandrosterone (DHEA) and other steroids. These studies and others [7] are consistent with the view that carbon isotope ratio methods could be very useful in discovering the origin of T and other steroids in an athlete's urine.

The present method for detecting T administration is finding a ratio of T to epitestosterone (T/E) in urine by GC–MS that exceeds six [8]. While T/E>6 is an excellent indicator of T administration, it is not a definitive test because a few individuals who have not used T have a T/E greater than six [9,10], and, other factors may alter T/E [11]. Further, since the median T/E is ~1 [12], individuals with low or normal T/E may take T and not exceed the reporting threshold of six, thus a negative report (T/E<6) does not exclude T administration. Lowering the T/E ratio threshold would result in detecting more cases of naturally elevated T/E, but would still allow some use of synthetic T. Other indirect means of detecting T administration have been discussed [13], but unlike $^{13}C/^{12}C$, none have the potential to serve as a direct indicator of T administration.

If a $^{13}C/^{12}C$ method is to be useful for increasing the sensitivity of detecting T administration, it is necessary to develop a screening method that is rapid, efficient, reliable, and requires a small volume of urine. In our first reports [2–4], the method required ~30 ml and it was relatively slow due to the high-performance liquid chromatography (HPLC) step. Furthermore, in the interest of understanding the method, we determined the $^{13}C/^{12}C$ of several compounds: cholesterol, DHEA, androst-5-ene-3β,17β-diol (T precursors), T itself, and T metabolites 5α-androstane-3α,17β-diol (5α-adiol) and 5β-androstane-3α,17β-diol (5β-adiol).

In this paper, we present a new sample preparation method that only requires 10 ml of urine, does not require HPLC fractionation, and provides chromatograms with excellent separation of the compounds of interest and virtually flat baselines.

2. Experimental

2.1. Urine samples

Urines were obtained from two groups of healthy male subjects. Subjects A–E were 20–59 years of age and were participating in a 25-week study concerned with the effects of T on behavior. The protocol was approved by the Harvard Medical School institutional review board. The subjects received intramuscular T enanthate (300 mg/week) or placebo, and urines were collected weekly for 26 weeks. Subjects F–J were participating in an 8-day study to discover the effects of T infusions on the hypothalamic–pituitary–testicular axis. After collection of the baseline urine, the subjects received an infusion of aqueous testosterone at doses of 7 mg/1.7m^2/24-h for days 1–2, 14 mg/1.7m^2/24-h for days 3–4, and 28 mg/1.7m^2/24-h for days 5–6. Timed urine samples were collected on each day. The protocol was approved by the Harbor-UCLA Hospital Ethics Committee.

2.2. Reagents for GC–C–IRMS sample preparation

The following steroids were obtained from

USADA 0770

Steraloids (Wilton, NH, USA): androst-5-ene-3β,17β-diol diacetate, 5β-androstane-3α,17β-diol diacetate, 5α-androstane-3α,17β-diol diacetate, 5α-androstane-3β,17β-diol diacetate, 5β-androstane-3β,17β-diol diacetate, 5β-pregnane-3α,20α-diol diacetate, 5β-pregnane-3α,17,20α-triol-3,20-diacetate, 5α-androstane-3α, 17α-diol, 5α-androstane-3β,17α-diol, 5α-pregnane-3β,20β-diol diacetate, 5α-pregnane-3α,20β-diol diacetate, androst-5-ene-3β,17α-diol diacetate, pregn-4-ene-3β,20α-diol, pregn-4-ene-3β,20β-diol, 5β-pregnane-3α,20β-diol diacetate, 5β-pregnane-3β,20α-diol diacetate, 5β-pregnane-3β, 20β-diol diacetate, 5α-pregnane-3α,20α-diol diacetate, pregn-5-ene-3β,20α-diol diacetate, 5α-androstane-3α,17β-diol and 5β-androstane-3β,17α-diol diacetate.

Sigma (St. Louis, MO, USA) provided 5α-pregnane-3β,20α-diol, and Boehringer Mannheim (Indianapolis, IN, USA) provided β-glucuronidase enzyme from *E. coli*. Solid phase extractions were carried out on Bakerbond solid-phase extraction columns filled with reverse phase octadecyl silica (J.T. Baker 7020-07 and 7020-13, Gallade Chemical, Fontana, CA, USA). All solvents and reagents were of analytical grade purity.

2.3. Overview of the analyses

Each urine underwent two different extraction and cleanup procedures and three different MS analyses. Firstly, our routine extraction procedure for anabolic steroids was performed followed by GC–MS [14]. This procedure estimated the T/E and the concentrations of T, E, 5β-adiol and 5α-adiol. Secondly, the new extraction and derivatization procedure was performed, followed by GC–MS to determine the identity and purity of peaks. Thirdly, the same extract was analyzed by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) to measure the carbon isotope ratio.

2.4. Routine steroid screen

The routine steroid screen includes addition of [1,2-$^2H_2$] testosterone to 2.5 ml of urine, liquid–solid extraction on a Bond-Elut C_{18} cartridge, enzymatic deconjugation with β-glucuronidase from *E. coli*, formation of trimethylsilyl derivatives, and GC–MS analysis in the selected ion monitoring mode as previously described [14]. Concentrations of T, 5β-adiol and 5α-adiol were estimated. All urine samples were found negative for exogenous anabolic androgenic steroids. As expected, the T/E of the post-T administration urines were greater than six (Table 1).

2.5. New steroid extraction for screening by GC–C–IRMS analysis

The steroids were extracted, purified, and acetylated as shown in Fig. 1. Urine (10 ml) was extracted by solid-phase extraction (SPE) on C_{18} column (500 mg), the conjugated steroids were eluted with methanol (8 ml) and the eluate evaporated to dryness. To hydrolyze the conjugated steroids, the methanolic residue was redissolved in 320 μl of 0.2 *M* phosphate buffer (pH 7.0), and 80 μl of β-glucuronidase was added as supplied. After incubation at 60°C for 1 h, the deconjugated steroids were extracted by SPE on C_{18} column (500 mg), by washing with 12 ml of methanol–water (20:80, v/v) and eluting with acetonitrile (12 ml). Acetylation of the extracts was carried out using pyridine (50 μl) and acetic anhydride (50 μl) for 1 h at 60°C incubation. The reaction medium was evaporated to dryness. The dried residue from acetylation was redissolved in 3 ml of acetonitrile–water (50:50, v/v). The steroid extract was purified by SPE on C_{18} column (1 g and 6 ml capacity). The fraction with the androstanediols and pregnanediols was obtained by elution with acetonitrile after washing with 12 ml of acetonitrile–water (50:50, v/v) and 15 ml of acetonitrile–water (75:25, v/v). The derivatized steroids were dried and dissolved in 25 μl of cyclohexane. This solution was analyzed by GC–MS and by GC–C–IRMS.

2.6. GC–MS analysis of acetylated derivatives

GC–MS analyses of a standard solution of acetylated derivatives of 5β-pdiol, 5β-adiol and 5α-adiol, and the acetylated derivatives from the extracted urines were performed on a Hewlett-Packard (HP) 5890 series II gas chromatograph equipped with an HP-50+ (50% phenyl–methylpolysiloxane) fused-silica capillary column (30 m×0.25-mm I.D., 0.15 μm film thickness) linked to an HP 5971 mass

Table 1
Summary of T/E and carbon isotope ratio ($\delta^{13}C‰$) data in subjects receiving weekly doses of testosterone enanthate for 7 weeks (subjects A–E) and data on urine samples collected from subjects receiving by infusion for 7 days (subjects F–J)

Subject	Urine	Collection Day	T/E	5β-pdiol		5β-adiol		5α-adiol		Difference	
				($\delta^{13}C$)‰	Mean	($\delta^{13}C$)‰	Mean	($\delta^{13}C$)‰	Mean	(5β-adiol)-(5β-pdiol)	(5α-adiol)-(5α-pdiol)
A	1	0	2.3	−25.91	−26.11	−26.77	−27.04	−25.12	−25.29	0.9	0.8
				−26.31		−27.31		−25.46			
	2	49	61.2	−25.27	−25.23	−33.07	−33.35	−29.00	−28.99	8.1	3.8
				−25.19		−33.63		−28.99			
B	3	0	0.4	−25.41	−25.33	−26.10	−26.23	−24.63	−24.64	0.9	0.7
				−25.25		−26.36		−24.65			
	4	28	19.7	−25.64	−25.72	−32.23	−32.20	−30.31	−30.27	6.5	4.5
				−25.81		−32.17		−30.23			
C	5	0	0.8	−24.96	−24.99	−24.85	−24.78	−24.34	−24.36	0.2	0.6
				−25.02		−24.70		−24.37			
	6	28	53.2	−24.65	−24.62	−30.86	−30.95	−30.87	−30.74	6.3	6.1
				−24.59		−31.04		−30.60			
D	7	0	1.1	−25.10	−25.13	−25.76	−25.64	−23.77	−23.68	0.5	1.4
				−25.16		−25.52		−23.59			
	8	28	22.3	−24.72	−24.60	−29.92	−29.97	−28.63	−28.53	5.4	3.9
				−24.48		−30.01		−28.42			
E	9	0	0.2	−24.16	−23.91	−25.80	−25.71	−25.65	−25.66	1.8	1.7
				−23.66		−25.62		−25.66			
	10	49	10.2	−23.32	−23.30	−30.11	−30.20	−30.96	−30.96	6.9	7.7
				−23.28		−30.28		−30.95			
F	11	0	1.5	−25.10	−25.14	−26.12	−26.07	−25.66	−25.67	0.9	0.5
				−25.17		−26.02		−25.67			
	12	6	11.7	−26.07	−26.11	−31.05	−31.06	−30.31	−30.32	5.0	4.2
				−26.14		−31.07		−30.32			
G	13	0	1.2	−25.61	−25.72	−25.81	−25.98	−24.59	−24.62	0.3	1.1
				−25.83		−26.15		−24.64			
	14	7	10.8	−26.10	−26.04	−31.00	−31.11	−29.69	−29.68	5.1	3.6
				−25.97		−31.22		−29.67			
H	15	0	1.6	−25.64	−25.65	−26.33	−25.46	−24.72	−24.79	0.2	0.9
				−25.65		−26.59		−24.85			
	16	5	15.4	−26.34	−26.17	−31.43	−31.27	−31.27	−31.24	5.1	5.1
				−25.99		−31.11		−31.21			
I	17	0	0.97	−25.42	−25.41	−26.71	−26.77	−27.25	−27.31	1.4	1.9
				−25.39		−26.83		−27.36			
	18	7	14.2	−24.30	−24.48	−28.37	−28.40	−28.99	−29.06	3.9	4.6
				−24.66		−28.43		−29.13			
J	19	0	0.91	−25.84	−25.79	−26.84	−26.83	−25.72	−25.61	1.0	0.2
				−25.74		−26.82		−25.50			
	20	5	11.4	−24.73	−24.78	−29.30	−29.35	−29.43	−29.47	4.6	4.7
				−24.82		−29.40		−29.51			

selective detector. The injections (2 μl) were made in the splitless mode using helium as carrier gas (76 kPa). The oven temperature was increased from 80°C (1 min) to 270°C (6 min) by 15°C/min, then to 300°C at 25°C/min, and maintained at the final temperature for 1 min.

USADA 0772

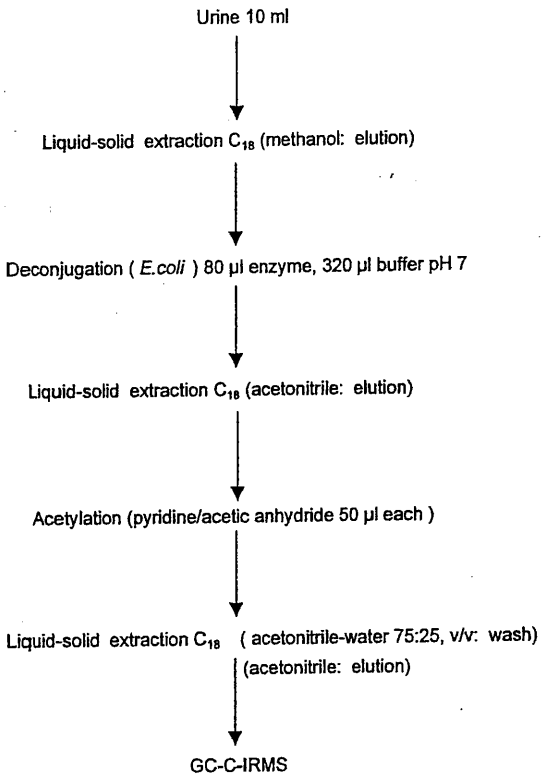


Fig. 1. Flow chart summarizing the sample preparation steps for screening urine samples by GC–C–IRMS.

2.7. GC–C–IRMS analysis

GC–C–IRMS analyses were performed on an HP 6890 gas chromatograph connected to a Micromass Isoprime isotope ratio mass spectrometer. The GC was equipped with an DB-17 column (J&W Scientific, 50%phenyl–methylpolysiloxane, 30 m×0.25-mm I.D., 0.25 µm film thickness). Helium was the carrier gas (76 kPa). Samples were injected in the splitless mode at 285°C. The GC separation began at 80°C for 1 min, then the oven temperature increased at 15°C/min to 270°C (6 min), then at 25°C/min to 300°C, and maintained at the final temperature for 1 min. The temperature of the combustion oven was 850°C and the oxidative catalyst was copper oxide pellets (0.5-mm thickness in a 6-mm quartz tube, 60 cm×65-mm I.D.)

The symbol δ is the standard notation for expressing carbon isotope ratios and it is defined as the relative difference in isotope ratio between the sample and an international standard (calcium carbonate from a fossil, *Belemnitella americana*) obtained from the Pee Dee formation in South Carolina (Pee Dee belemnite or 'PDB') [15] and relatively rich in ^{13}C, calculated as:

$$\delta^{13}C‰ = \frac{R_{sample} - R_{PDB}}{R_{PDB}} \times 1000$$

where R_x refers to the $^{13}C/^{12}C$ of the sample or international standard and is represented by $^{13}C‰$ ('per mil'). Each extract was injected twice into the GC–C–IRMS and the replicate measurements were averaged.

3. Results and discussion

A principal objective of the new sample preparation scheme presented here was to develop a rapid method for screening urine samples by GC–C–IRMS. To accomplish this, it was necessary to eliminate the HPLC purification step from our previous methods [2–4]. Secondly, we aimed to reduce the sample volume requirements from ~30 to 10 ml or less. Thirdly, a screening method must be rapid and efficient, therefore we wished to adapt the method to batch processing in order to improve sample analysis time and sample throughput. A flowchart summarizing the procedure is shown in Fig. 1. The principal features are three successive liquid–solid extractions, deconjugation with *E. coli* β-glucuronidase after the first extraction, acetylation after the second extraction, and a final extraction of the acetates.

3.1. Chromatography

Carbon isotope ratio measurements require rigorous attention to detail during sample preparation because impurities can result in spurious $\delta^{13}C‰$ values. Chromatographic resolution is the most critical parameter because the isotopic measurement of the peaks of interest are altered by coeluting or poorly separated peaks. Figs. 2 and 3 show the characteristics of the GC–MS and GC–C–IRMS chromatograms of our procedure. Fig. 2 shows a flat baseline and enough resolution of the three peaks of

USADA 0773

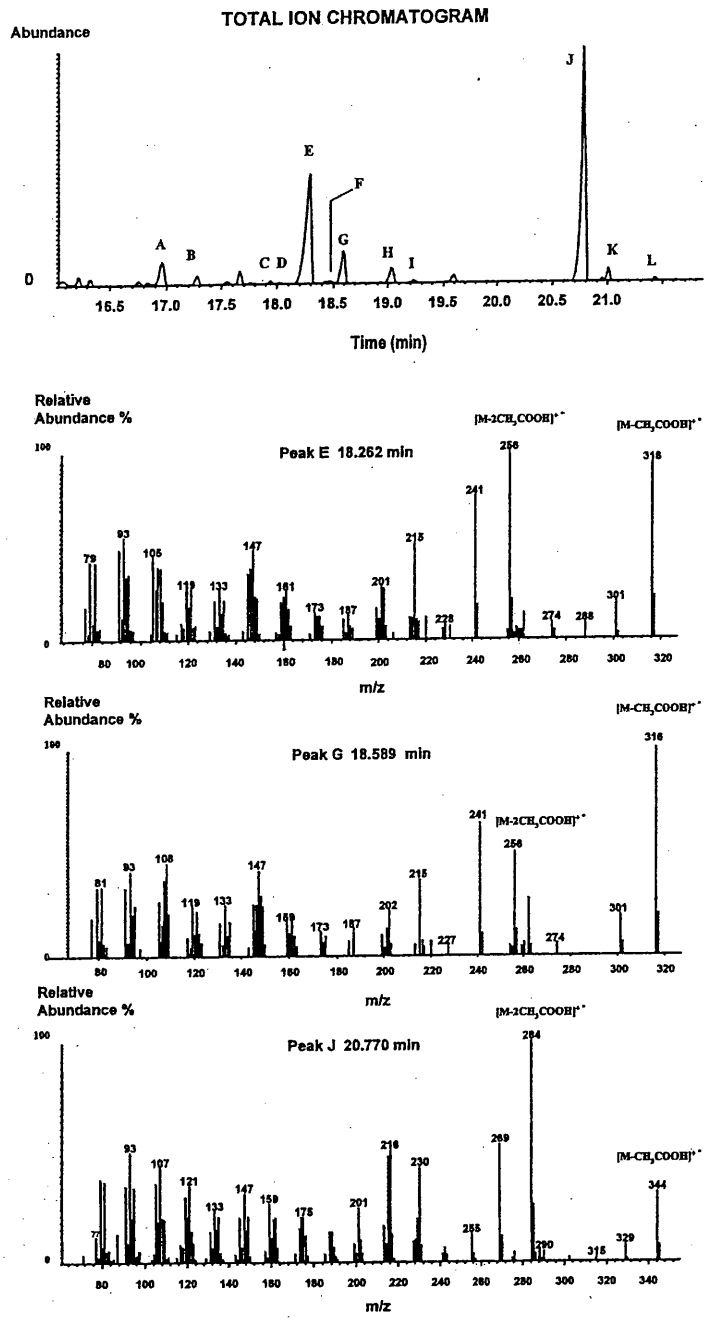


Fig. 2. Total ion chromatogram, mass spectra of compounds used in GC–MS analysis. A and B = unidentified androstene. C = 5β-androstane-3α,17α-diol diacetate. D = unidentified androstene. E = 5β-androstane-3α,17β-diol diacetate (5β-adiol-diacetate). F = 5α-androstane-3α,17α-diol diacetate and androst-5-ene-3α,17β-diol diacetate. G = 5α-androstane-3α,17β-diol diacetate (5α-adiol-diacetate). H = androst-5-ene-3β,17α-diol diacetate. I = androst-5-ene-3β,17β-diol diacetate. J = 5β-pregnane-3α,20α-diol diacetate (5β-pdiol-diacetate). K = 5α-pregnane-3α,20α-diol diacetate. L = pregn-5-ene-3β,20α-diol diacetate.

USADA 0774

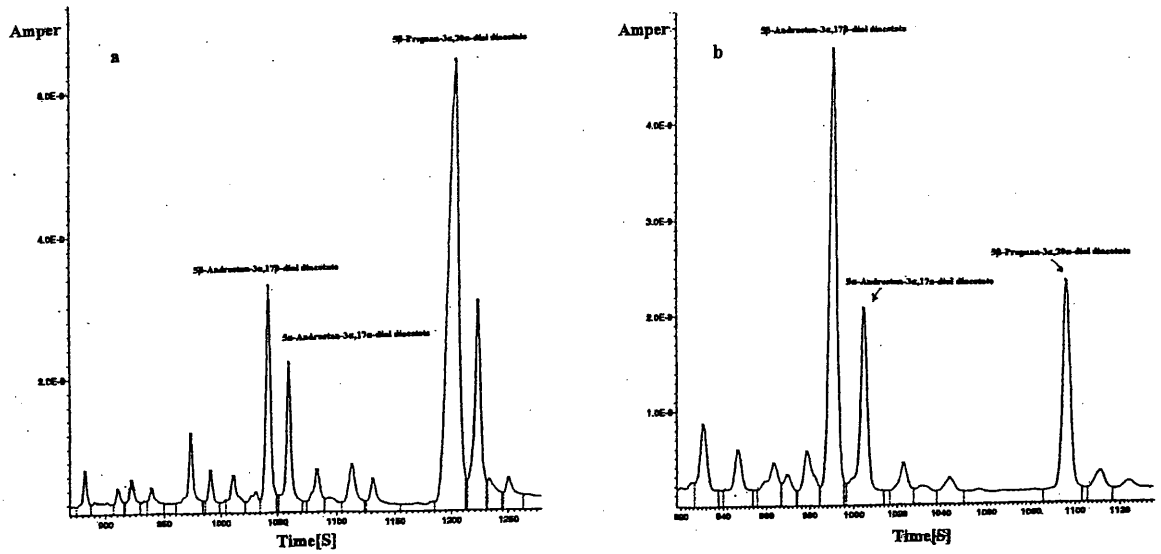


Fig. 3. GC–C–IRMS chromatograms from a negative (a) and a positive (b) urine.

interest. Fig. 3 also has a flat baseline and background characteristic of GC–C–IRMS. Examples of the spectra of the peaks of interest and their retention times are shown in Fig. 2.

The characteristics of the chromatograms were optimized by three successive SPE extractions. In the first step, the conjugated steroids were extracted using C_{18}-SPE [16]. Following enzymatic hydrolysis, we found that a second SPE step gave higher recoveries compared to liquid extraction. In addition, the second SPE step substantially improved the quality of the chromatograms, and it was accomplished with a minimum volume of elution solvent (12 ml).

In our previous method [3,4] the acetates were purified by HPLC, a procedure which inevitably carries some risk of isotopic discrimination. In the new procedure it is important to show that discrimination does not occur during the final acetonitrile–water (75:25, v/v) wash. To accomplish this we determined the $\delta^{13}C‰$ values on the diacetates of 5β-pregnane-3α,20α-diol, 5β-androstane-3α,17β-diol and 5α-androstane-3α,17β-diol after washing with 15 and 21 ml of acetonitrile–water (75:25, v/v). In order to ensure a range of $\delta^{13}C‰$ values and heterogeniety, we studied eight urines obtained from four subjects, before and after T administration. As shown in Table 2, the wash volume did not significantly influence the $\delta^{13}C‰$ values of the diacetates ($p=0.2$, before and $p=0.06$, after). In addition, we noted that the 15 ml wash eliminated the underivatized and monoacetylated steroids, while the 21 ml wash resulted in some loss of androstanediol diacetates. The underivatized keto steroids are also

Table 2
Effect of the volume wash of acetonitrile–water (75:25, v/v) on the carbon isotope ratio of diacetylated steroids 5β-pdiol, 5α-adiol, and 5β-adiol in four pre-T and four post-T urine at the third SPE clean-up

Urine	Compound	Volume of Wash (ml)			
		15	21	15	21
		Pre-T urine		Post-T urine	
1	5β-pdiol	−25.82	−25.70	−25.36	−25.64
2		−26.11	−25.12	−24.78	−24.69
3		−25.14	−24.65	−24.48	−24.07
4		−25.07	−23.09	−24.62	−24.62
1	5β-adiol	−25.90	−25.56	−31.60	−30.95
2		−27.04	−26.09	−30.95	−32.58
3		−24.36	−23.52	−28.40	−30.94
4		−25.08	−26.88	−31.11	−33.10
1	5α-adiol	−25.48	−25.34	−28.91	−29.24
2		−26.46	−23.52	−30.74	−31.99
3		−25.29	−26.38	−29.67	−29.02
4		−25.62	−26.07	−29.06	−31.53

USADA 0775

eliminated during the first 12 ml wash with acetonitrile–water (50:50, v/v).

3.2. Analysis time and urine volume

In our previous publications, the HPLC step was rate-limiting with the result that a batch of 10 samples required nearly 2 days to process [2–4]. The new method enables a batch of 10 samples to be extracted and analyzed by GC–C–IRMS analysis in 8 h. In our previous publications [2–4], it was necessary to use 30 ml of urine in order to obtain satisfactory estimates of the $^{13}C‰$ of T. The typical male urine received in our doping control laboratory contains ~40 ng/ml of T, whereas the median concentrations of 5β-adiol and 5α-adiol are 121 and 68 ng/ml, respectively. These concentrations were determined by summarizing our database of ~10 000 male urines. The higher concentration of the diols enables the new analysis to be carried out on 10 ml of male urine. We determined the concentration of the diols from the GC–MS screening analysis, then selected a sample volume that would provide approximately 700 ng of each diol.

3.3. Choice of enzyme for deconjugation

The three diols of interest are excreted as both glucuronide and sulfate conjugates [17], therefore the specific advantages and disadvantages of deconjugating with β-glucuronidase from *E. coli* or *H. pomatia* are considered. The *H. pomatia* enzyme gives a greater yield of sulfates; however, the 3 h hydrolysis time is a distinct disadvantage for a rapid screening method. *E. coli* enzymatic hydrolysis takes only 1 h due to a high specific activity, which provides chromatograms with excellent separation of 5β-adiol, 5α-adiol, and 5β-pdiol from surrounding peaks. In addition, when *H. pomatia* was used to deconjugate, the chromatograms were more complex because several isomers of the diols of interest were detected.

3.4. Selection of T metabolites and of andogenous reference

Following a dose of T, only 1–2% is excreted as T conjugates and another 4–5% is excreted as 5β-adiol and 5α-adiol [17–19]. Some dihydrotestosterone and other minor metabolites of T are detected in human urine but the concentrations are too low for carbon isotope ratio measurements. The majority of administered T is excreted as androsterone and etiocholanolone. These two steroids are present in large amounts (1–3 μg/ml); however, they are the final elimination path for many steroids, therefore the effect of T administration on their $^{13}C‰$ values is diluted. Although we are investigating the $^{13}C‰$ of androsterone and etiocholanolone, at the present time the optimal metabolites of T for $^{13}C‰$ analysis are 5β-adiol and 5α-adiol.

The endogenous reference concept is important to the analysis because the selected compound serves as an internal control. The $^{13}C‰$ of the endogenous reference reflects the diet of the individual and will vary among individuals [2–5]. By using an endogenous reference, one can better estimate the degree of incorporation of exogenous T into T and its metabolites. Furthermore, one can calculate derived variables such as the ratio of $^{13}C‰$ of endogenous reference/metabolite or the difference between the $^{13}C‰$ of the endogenous reference and metabolite.

In our previous studies, we determined the $^{13}C‰$ of T precursors cholesterol acetate, androst-5-ene-3β,17β-diol diacetate, and dehydroepiandrosterone acetate. We have abandoned cholesterol because the origin of urinary cholesterol is not well understood. Dehydroepiandrosterone acetate and androst-5-ene-3β,17β-diol diacetate provide satisfactory $^{13}C‰$ measurements [2,3]; however, they are not present in sufficient quantities after deconjugation with *E. coli*. In the present assay, the recovery of 5β-pdiol is high and its chromatographic properties are excellent. In addition, Shackleton et al. report that the $^{13}C‰$ of 5β-pdiol is not affected by T administration and they find it to be a good internal reference standard [5].

3.5. Detection of testosterone administration

A principal objective of this work was to determine if our new method further confirms the utility of $^{13}C‰$ measurements in detecting T administration. The T/E of the pre-T urines were all less than 2.3 (mean=1.1, range=0.2–2.3). The mean post-T urine T/E was 23 (range=10.2–61). These data confirm that T administration increases

USADA 0776

T/E and that the samples are suited for determining the effect of T administration on the $\delta^{13}C‰$ of andogenous reference and metabolites.

To strengthen our confidence in the method, the analyst conducting the GC–C–IRMS and data reduction was blind to the content of the samples and had no knowledge of the clinical protocol. Table 1 shows the T/E and $\delta^{13}C‰$ values for 10 subjects who submitted pre-T and post-T urines. Subjects A–E received T enanthate (300 mg/week) and the post-T urines were collected in the 4th or 7th week of weekly T administration, and subjects F–J received T by intravenous infusion and the post-T urine was collected on days 5–7. Following T administration, the $\delta^{13}C‰$ values for both diols decreased. The mean pre-T and post-T $\delta^{13}C‰$ values for 5β-adiol were −26.2 (C.V.=2.6%) and −30.8 (C.V.=5.6%), respectively. This difference was significant at $p<0.001$. Likewise the mean pre-T and post-T $\delta^{13}C‰$ values for 5α-adiol were −25.2 (C.V.=3.9%) and −29.9 (C.V.=3.1%), respectively ($p<0.001$). The magnitude of the mean decrease was 4.6 for 5β-adiol and 4.7 for 5α-adiol. These findings confirm that the

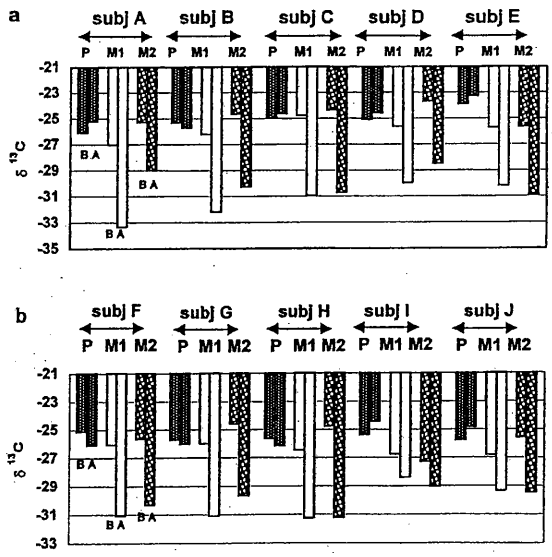


Fig. 4. Mean carbon isotope ratio values ($\delta^{13}C‰$) of pregnanediol (P=5β-pdiol), and T metabolites (M1=5β-adiol), (M2=5α-adiol) in five subjects who received T enanthate (3a) and five subjects who received T by infusion (3b). Pre-T $\delta^{13}C‰$ values are labeled before (B) and post-T $\delta^{13}C‰$ values are labeled after (A).

$\delta^{13}C‰$ of T metabolites decreases following T administration.

As expected, the carbon isotope ratio for 5β-pdiol was not affected by exogenous T administration. The mean pre-T and post-T $\delta^{13}C‰$ values were −25.3 (C.V.=2.4%) and −25.1 (C.V.=3.7%), and these values were not significantly different ($p=0.34$). These results concur with those obtained by Shackleton et al. [5], and confirm that the $\delta^{13}C‰$ of T precursors are not affected by T administration. In addition, the $\delta^{13}C‰$ values for 5β-pdiol were similar to the values observed in our previous work for other T precursors: cholesterol (−24.6‰), DHEA (−24.3‰), and androst-5-ene-3β,17β-diol (−26.2‰) [2–4].

Fig. 4a and b summarize the data such that the consistency of the pre-T $\delta^{13}C‰$ measurements and the magnitude of the change in the post-T samples are apparent. The pre-T and post-T samples are shown as pairs with the pre-T $\delta^{13}C‰$ on the left, and the post-T values on the right (indicated with arrows for subj A). It is noteworthy that the magnitude of the post-T change in the $\delta^{13}C‰$ of 5β-adiol and 5α-adiol in subjects I and J (Fig. 4b) is less than that of the other subjects. These two subjects were the only Asian–Americans in the study and there is some evidence that Asians may differ from non-Asians in the magnitude of the effect of exogenous T on T/E [5].

3.6. Additional markers of T administration

To further elucidate the relationship between the $\delta^{13}C‰$ of pregnanediol and androstanediols we calculated two derived variables, namely the differences in $\delta^{13}C‰$ between 5β-adiol and 5β-pdiol and between 5α-adiol and 5β-pdiol. As shown in Table 1, the maximum difference in (5β-adiol)–(5β-pdiol) and (5α-adiol)–(5β-pdiol) in urines obtained pre-T were 1.8 and 1.9, respectively; these pre-T values should be compared to the minimum post-T differences which are 3.9 and 3.6, respectively. The maximum post T differences were 8.2 and 7.7, respectively. A t-test (paired) on the pre-T and post-T difference scores for (5β-adiol)–(5β-pdiol) was significant ($p<0.001$). Similarly the (5α-adiol)–(5β-pdiol) difference scores were significant ($p<0.001$). This confirms that the difference between the $\delta^{13}C‰$

USADA 0777

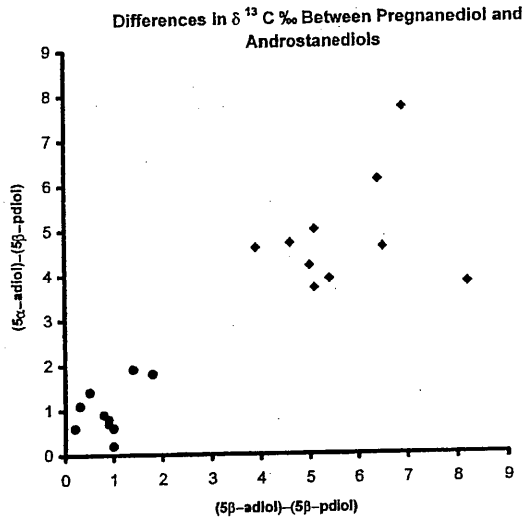


Fig. 5. Testosterone metabolite-pregnanediol $\delta^{13}C‰$ values of pre-T (●) and post-T (♦) urines collected from 10 subjects. The *X*-axis is the difference between 5β-adiol and 5β-pdiol (5β-adiol)–(5β-pdiol) and the *Y*-axis is the difference between 5α-adiol and 5β-pdiol (5α-adiol)–(5β-pdiol).

values for the andogenous reference and metabolites accurately discriminate between urines collected before and after T administration. Fig. 5 plots these difference scores for the pre-T and post-T urines. The values on the pre-T urines cluster in the lower left and all values are less than 1.9. The post-T urines all have difference scores that exceed 3.6.

The ratio of $^{13}C/^{12}C$ values of the androstanediols (AD) to pregnanediol (PD) has been reported to be a useful indicator of T administration [5]. Accordingly, we calculated the mean $^{13}C/^{12}C$ of the two androstanediols (5α-adiol+5β-adiol)/2 and the ratio (5α-adiol+5β-adiol)/2*5β-pdiol (AD/PD). The range of AD/PD $^{13}C/^{12}C$ values for the pre-T urines is 0.981–1.074, which is in excellent agreement with the maximum baseline value of 1.08 reported by Shackleton et al. [5]. Further, the AD/PD ratios in the post-T urines (range 1.167–1.312) were all below 1.1, the value suggested by Shackleton et al. [5] to distinguish between T users and non-users.

As far as the acetate group's contribution is concerned the $\delta^{13}C‰$ values that we obtained are very close to those obtained by Shackleton et al. [5] and Aguilera [20] even though different source batches of acetic anhydride were used. Finally, the conclusions concerning a negative or positive case are not affected by the discrepancies between derivatized and free compounds as they remain consistent within a normal range variation. In addition, the contribution of the acetate group is the same for the endogenous references and the testosterone metabolites.

4. Conclusions

We propose a new screening method for the detection of exogenous testosterone administration using GC–C–IRMS. The method meets the requirements for use in routine screening. Firstly, only 10 ml of male urine was required to obtain satisfactory estimates of $\delta^{13}C‰$. Secondly, by replacing the HPLC step in our previous procedure with three successive solid-phase extractions, greater speed and efficiency was achieved by processing samples in batches. Thirdly, the choice of deconjugating with β-glucuronidase from *E. coli* rather than from *H. pomatia* resulted in high quality chromatograms with excellent resolution of the peaks of interest. The method gives consistent and reproducible $\delta^{13}C‰$ values with a mean C.V. for replicates of 1.6%. We confirmed that T administration results in a decrease in the $\delta^{13}C‰$ values of T metabolites, and we found significant differences between the $\delta^{13}C‰$ values of (5β-pdiol)–(5β-adiol) obtained on the pre-T and post-T urines, and between the (5β-pdiol)-(5α-adiol) of the pre-T and post-T urines.

We propose that the method described herein be used to supplement the T/E results obtained GC–MS. In the case of an elevated T/E and normal $\delta^{13}C‰$ values there would be no need to notify the athlete that additional testing is necessary. The sample would be reported negative. With further experience, we anticipate that samples with low $\delta^{13}C‰$ values and T/E>6 could be reported positive for T. Finally, the method may detect cases of T administration characterized by low $\delta^{13}C‰$ values and a T/E that is less than six.

Acknowledgements

We are grateful for financial support from the National Collegiate Athletic Association, the National Football League, and the United States Olympic Committee. K. Schramm skillfully assisted with the

USADA 0778

analyses, and T. Callahan and J. Wilson provided outstanding editorial assistance.

References

[1] G. Southan, A. Mallet, J. Jumeau, S. Craig, N. Poojara, D. Mitchell, M. Wheeler, R.V. Brooks, Programme and Abstracts of the second International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona, (1990) 306.
[2] M. Becchi, R. Aguilera, Y. Farizon, M.M. Flament, H. Casabianca, P. James, Rapid Commun. Mass Spectrom. 8 (1994) 304.
[3] R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic, H.G. Pope, J. Mass Spectrom. 31 (1996) 169.
[4] R. Aguilera, M. Becchi, C. Grenot, H. Casabianca, C.K. Hatton, J. Chromatogr. B 687 (1996) 43.
[5] C.H.L. Shackleton, A. Phillips, T. Chang, Y. Li, Steroids 62 (1997) 379.
[6] C.H.L. Shackleton, E. Roitman, A. Phillips, T. Chang, Steroids 62 (1997) 665.
[7] S. Horning, H. Meyer, M. Machnik, W. Shänser, A. Hilkert, J. Oeßelmann, in: W. Shänser, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, SPORT und BUCH Strauß, Cologne (1997) 275.
[8] M. Donike, K.R. Bärwald, K. Klostermann, W. Schänzer, J. Zimmerman, in: H. Heck, W. Hollman, H. Liesen, R. Rost (Eds.), Sport: Leistung Und Gesundheit, Deutscher Ärzte-Verlag, Köln, Germany, 1982, p. 293.
[9] E. Raynaud, M. Audran, J.F. Brun, C. Fedou, J.L. Chantal, A. Orsetti, Lancet 340 (1992) 1468.
[10] L. Dehennin, J. Endocrinol. 142 (1994) 353.
[11] D.H. Catlin, C.K. Hatton, S.H. Starcevic, Clin. Chem. 43 (1997) 1280.
[12] M. Garle, R. Ocka, E. Palonek, I. Björkhem, J. Chromatogr. B 687 (1996) 55.
[13] D.H. Catlin, D.A. Cowan, Clin. Chem. 38 (1992) 1685.
[14] D.H. Catlin, R.C. Kammerer, C.K. Hatton, M.H. Sekera, J.M. Merdink, Clin. Chem. 33 (1987) 319.
[15] P. Deines, in: P. Fritz, J.C. Fontes (Eds.), Handbook of Environmental Isotope Geochemistry, Elsevier, New York, 1980, p. 329.
[16] R. Massé, C. Ayotte, R. Dugal, J. Chromatogr. 489 (1989) 23.
[17] L. Dehennin, M. Ferry, P. Lafarge, G. Pérès, J.P. Lafarge, Steroids 63 (1998) 80.
[18] R. Horton, J.M. Rosner, P.H. Forsham, Proc. Soc. Exp. Biol. Med. 114 (1963) 400.
[19] A.M. Camacho, C.J. Migeon, J. Clin. Invest. 43 (1964) 1083.
[20] R. Aguilera, Ph.D. Thesis, Centre National de la Recherche Scientifique, France, 1996.

USADA 0779

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. **14**, 2294–2299 (2000)

A rapid screening assay for measuring urinary androsterone and etiocholanolone $\delta^{13}C$ (‰) values by gas chromatography/combustion/ isotope ratio mass spectrometry

Rodrigo Aguilera[1]*, Thomas E. Chapman[1] and Don H. Catlin[1,2]

[1]UCLA Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology, University of California at Los Angeles, 2122 Granville Avenue, Los Angeles, CA 90025-6106, USA
[2]Department of Medicine, University of California at Los Angeles, 2122 Granville Avenue, Los Angeles, CA 90025-6106, USA.

A gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) method is described and validated for measurement of $\delta^{13}C$ values of the acetate derivatives of urinary etiocholanolone and androsterone. The analysis was performed with only 2 mL of urine. The sample preparation consisted of deconjugation with β-glucuronidase, solid phase extraction, and derivatization with acetic anhydride and pyridine. The within-assay precision of two quality control (QC) urine samples ranged from 0.5 to 2.1 CV%. The between-assay precision in the same QC urines ranged from 1.7 to 3.4 CV%. Administration of testosterone enanthate to a subject resulted in a 6‰ decrease in $\delta^{13}C$ values from −25‰ (baseline) to −31‰. Two weeks after testosterone administration was discontinued, the $\delta^{13}C$ values remained abnormally low while the urine testosterone/epitestosterone (T/E) ratio returned to less than 6. This relatively simple method is useful for rapidly screening a large number of urine samples, including those with T/E <6. Copyright © 2000 John Wiley & Sons, Ltd.

Received 13 September 2000; Revised 13 October 2000; Accepted 14 October 2000

Detecting doping with endogenous androgens has long been an analytical challenge because the main technique in use, gas chromatography/mass spectrometry (GC/MS), cannot distinguish pharmaceutical from endogenous androgens. For this reason, testosterone doping is detected by measuring the testosterone/epitestosterone (T/E) ratio by GC/MS in the ~100 000 urine samples collected annually by the doping authorities.[1] In less than 1% of all cases, the screen finds T/E ratios >6. Some of these cases are T users and others have naturally, chronically elevated T/E.[2] The current procedure for determining which are users and which are non-users is costly and time consuming. Typically the authorities conduct a T/E versus time profile of past samples from the same athlete or obtain additional samples for T/E analysis. The authorities may commission or recommend an endocrine evaluation of the athlete. These steps are needed before a doping infraction is declared, because rare individuals will have naturally elevated T/E, and other drugs and conditions may influence the urine T/E.[3]

In the last six years, isotope ratio mass spectrometry (IRMS) methods have helped enormously with the complex problem of discerning androgen users from non-users. The methods capitalize on the difference in $^{13}C/^{12}C$ ratio between pharmaceutical T and endogenous human T and, therefore, between its precursors and metabolites.[4–8] GC/C/IRMS methods have been used successfully to detect doping with T,[5–7] dehydroepiandrosterone (DHEA),[8] and dihydrotestosterone (DHT).[9,10]

While IRMS methods have proved extremely useful in detecting the administration of exogenous androgens, one disadvantage is their limited capacity to process a large number of samples in a short time. Current GC/C/IRMS methods are not optimized for rapid screening as they are labor-intensive, require large sample volumes, and often require preliminary sample clean-up by high performance liquid chromatography. This paper describes a new rapid GC/C/IRMS screening method based on determining the $\delta^{13}C$ values of the acetate derivatives of the etiocholanolone and androsterone extracted from 2 mL of urine.

EXPERIMENTAL

Urine samples

Urine samples were obtained from two healthy male subjects, age 29 and 52, from a 24-week study of the effects of T on behavior. Both subjects received weekly injections: placebo in weeks 1–13 and 20–24, and T enanthate in weeks 14–15 (150 mg), in weeks 16–17 (300 mg), and in weeks 18–19 (600 mg). One urine from one of the two subjects was selected to be the positive quality control urine (QC-Pos). Nine urines from the other subject were analyzed. The protocol was approved by the Harvard Medical School institutional review board and has been previously described.[11] The negative quality control urine (QC-Neg)

*Correspondence to: R. Aguilera, UCLA Olympic Analytical Laboratory, 2122 Granville Avenue, Los Angeles, CA 90025-6106, USA.
E-mail: rodrigoa@ucla.edu
Contract/grant sponsor: National Collegiate Athletic Association.
Contract/grant sponsor: The National Football League.
Contract/grant sponsor: United States Olympic Committee.

Copyright © 2000 John Wiley & Sons, Ltd.

USADA 0780

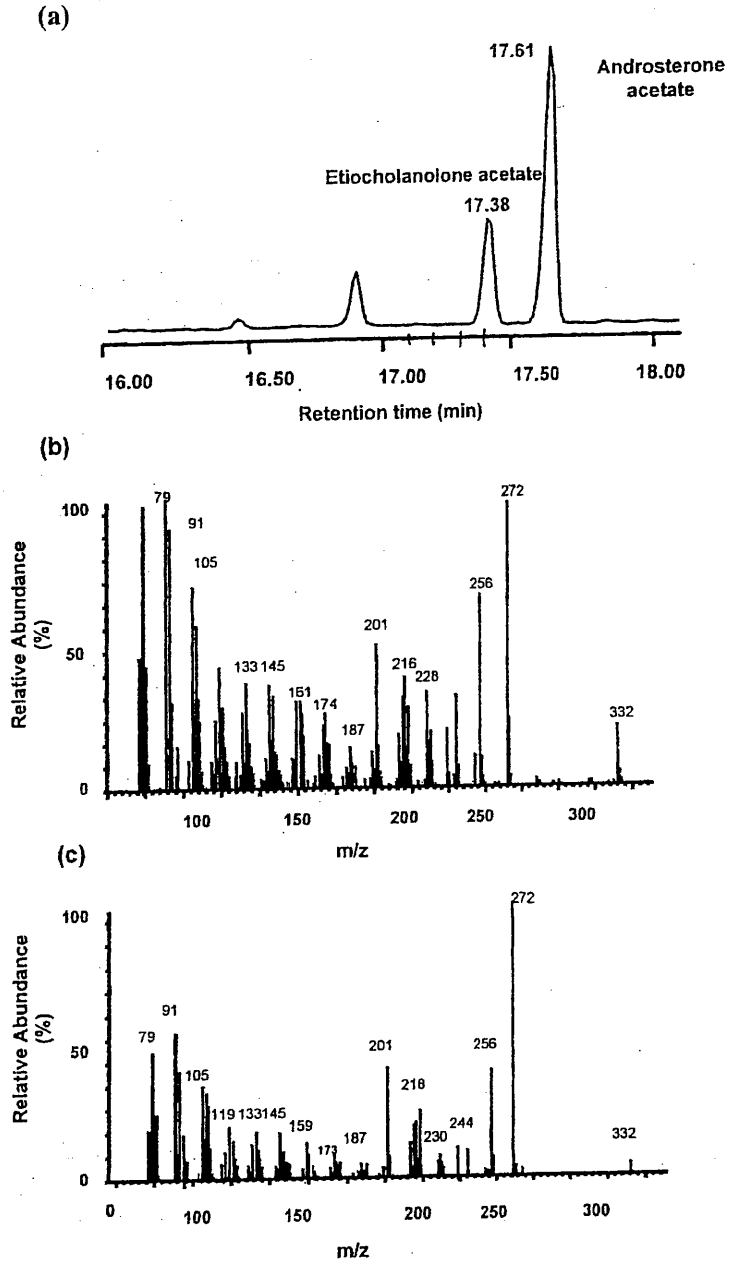


Figure 2. GC/MS total ion current chromatogram and mass spectra of etiocholanolone and androsterone (acetates) from 2 mL urine. (a) Total ion current chromatogram (*m/z* 75 to 600). (b) Mass spectrum of etiocholanolone acetate (17.38 min). (c) Mass spectrum of androsterone acetate (17.61 min).

control chart, which was updated with each successive assay. Beginning with the fifth assay, the standard deviations (SDs) of the $\delta^{13}C$ values obtained in all previous assays were used to determine if the latest assay was acceptable. If more than one of the four SDs were outside the ±2 SD limit the assay was repeated. The within-assay precision was determined by preparing five aliquots of QC-Pos and QC-Neg in the same batch and injecting each once. The between-assay precision was determined by extracting one aliquot of QC-Pos and QC-Neg per day for 25 days, and injecting each once.

RESULTS

GC/MS identification and purity check of human urine extracts

A total ion current chromatogram of a derivatized urine extract is shown in Fig. 2(a) and the mass spectra of etiocholanolone and androsterone acetate from this extract are shown in Figs 2(b) and 2(c). Both have a molecular ion of *m/z* 332 and prominent fragment ions of *m/z* 256 and 272. The relative abundances of the main ion ratios in the

Copyright © 2000 John Wiley & Sons, Ltd.

USADA 0782

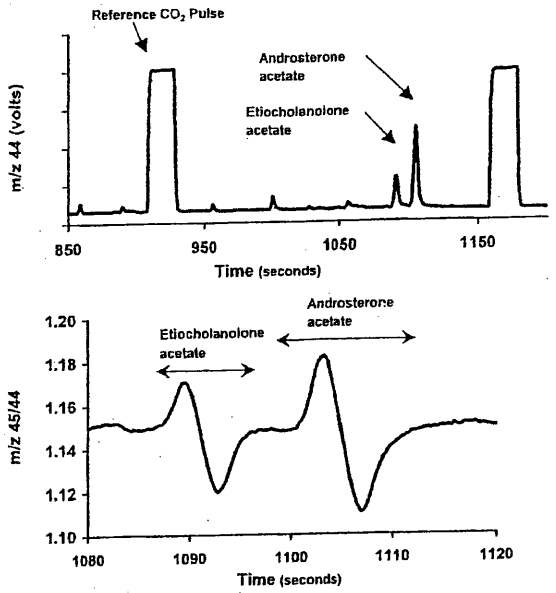


Figure 3. GC/C/IRMS chromatograms of *m/z* 44 (upper), and ratio of *m/z* 45/44 from 1080 to 1120 s (lower).

compounds extracted from urine were within 20% of those of standards. The chromatogram shows symmetrical peaks for androsterone and etiocholanolone acetate, no tailing, and no evidence of co-eluting compounds.

GC/C/IRMS characteristics of etiocholanolone and androsterone acetate

A typical GC/C/IRMS trace of *m/z* 44 is illustrated in Fig. 3(a). Reference carbon dioxide gas pulses (20 s width) were introduced at 900 and 1150 s. The pulse at 900 s was used to normalize the $\delta^{13}C$ values. The acetylated derivatives of etiocholanolone and androsterone eluted at 1090 and 1105 s, respectively (18.2 and 18.4 min). The 45/44 ion ratio is illustrated in Fig. 3(b). The carbon dioxide peak shapes were symmetrical with no tailing. The etiocholanolone and androsterone acetate peaks were baseline resolved (Figs 3(a) and 3(b)).

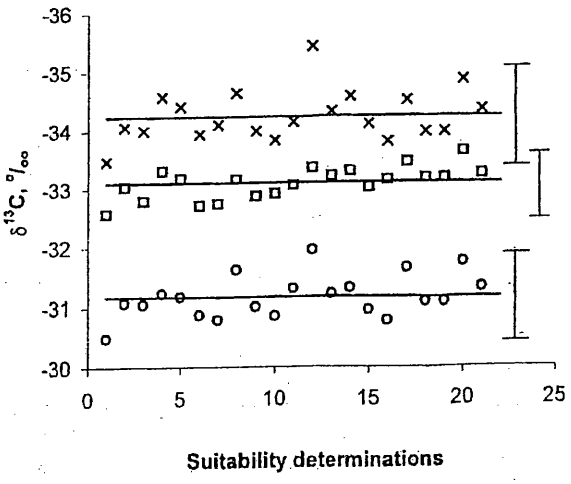


Figure 4. Quality control chart for the alkane mixture showing horizontal lines (——) for the means of C_{14} (□), C_{15} (○), and C_{16} (×). The vertical bars indicate ± 2SD.

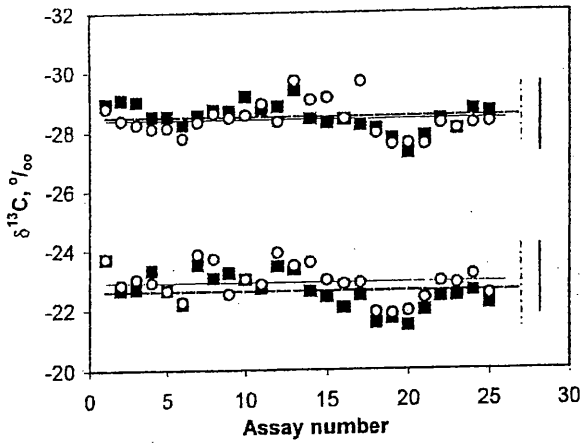


Figure 5. Quality control chart for the QC-Neg (lower) and the QC-Pos (upper) showing the $\delta^{13}C$ values for acetylated androsterone (□) and etiocholanolone (○). The means (horizontal lines) and the ±2 SD (vertical bars) are indicated by (----) for androsterone acetate and by (——) for etiocholanolone acetate.

Systems suitability, quality control, and precision

After 21 system suitability determinations over 5 months the overall means for the C_{14}, C_{15}, and C_{16} alkanes were −33.1, −31.2, and −34.2‰, respectively (Fig. 4). These 21 system suitability determinations provided SDs of 0.26, 0.35, 0.43‰, for the C_{14}, C_{15}, and C_{16} alkanes, respectively, and the corresponding coefficients of variation (CVs) were 0.78, 1.14, and 1.25%.

A GC/C/IRMS assay sequence consisted of a water blank, the QC-Neg, the QC-Pos, and up to 30 samples. After 25 assays over 5 months the overall means for etiocholanolone and androsterone acetate in the QC-Neg were −22.9 and −22.6‰, respectively, while for the QC-Pos the means were −28.4 and −28.5‰, respectively (Fig. 5). For etiocholanolone the SDs of the 25 assays were 0.59 and 0.57‰ for the QC-Neg and QC-Pos, respectively, and the corresponding CVs were 2.6 and 2.0%. For androsterone the SDs of the 25 assays were 0.61 and 0.48‰ for the QC-Neg and QC-Pos, respectively, and the corresponding CVs were 2.7 and 1.7%.

The descriptive statistics for the within-assay precision experiment on QC-Neg and QC-Pos are shown in Table 1. For QC-Neg the SDs were 0.38 and 0.46‰ for androsterone and etiocholanolone acetate, respectively, and the corresponding CVs were 1.8 and 2.1%. For the QC-Pos the SDs were 0.17‰ for androsterone acetate and 0.15‰ for etiocholanolone acetate, the corresponding CVs were 0.6% and 0.5%.

The between-assay SDs for QC-Neg were 0.78 and 0.73‰ for androsterone and etiocholanolone acetate, respectively, and the corresponding CVs were 3.4 and 3.2% (Table 2). For QC-Pos the SD values were 0.49 and 0.56‰ and the CVs were 1.7 and 2.0% for androsterone and etiocholanolone acetate, respectively. The mean $\delta^{13}C$ values for androsterone and etiocholanolone acetate in the

Copyright © 2000 John Wiley & Sons, Ltd.

USADA 0783

Table 1. Within-assay precision. Five aliquots of the QC-Pos and the QC-Neg were extracted, derivatized and assayed on the same day

	QC-Neg δ^{13}C (‰)		QC-Pos δ^{13}C (‰)	
Sample	Etio	Andro	Etio	Andro
1	−21.3	−20.6	−28.3	−28.3
2	−21.3	−21.4	−28.3	−28.3
3	−22.3	−21.2	−28.5	−28.4
4	−22.2	−21.7	−28.7	−28.7
5	−21.9	−21.4	−28.5	−28.5
Mean	−21.8	−21.3	−28.4	−28.4
SD	0.46	0.38	0.15	0.17
CV%	2.1	1.8	0.5	0.6

Etio = Etiocholanolone acetate
Andro = Androsterone acetate

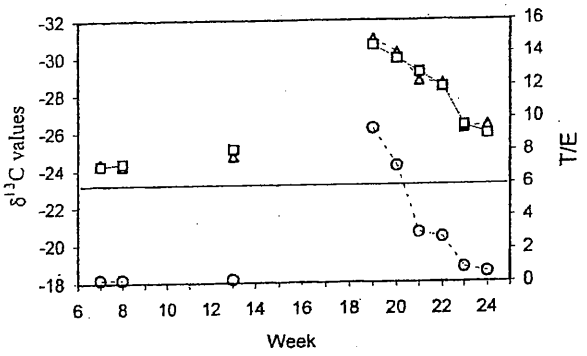


Figure 6. Time-profile of T/E ratio (○), and androsterone (■) and etiocholanolone (△) acetate δ^{13}C values after administration of T enanthate 100 mg in weeks 14–15, 300 mg in weeks 16–17, and 600 mg in weeks 18–19. Horizontal line is T/E = 6.

QC-Neg were −22.5 and −22.7‰, respectively. As expected, the mean δ^{13}C values for androsterone and etiocholanolone acetate in the QC-Pos were lower, −28.4 and −28.2‰.

Assay throughput

A batch of 30 samples can be prepared for IRMS analysis in about 2 h. The GC/C/IRMS is set up during the hydrolysis step and the IRMS analysis begins at the end of the 8-h work day. The batch IRMS run-time is about 12 h. Data reduction and reporting is performed in about 3 h, thus about 30 samples can be analyzed and reported in 24 h. The total time to analyze a single sample is ~10 h.

δ^{13}C values following testosterone administration

Figure 6 shows the baseline and post-T δ^{13}C values and T/Es for the subject who received 150–600 mg/week of T enanthate. Note that during weeks 14–18 of T administration no data were available. During three pre-T weeks (7, 8, and 13) the mean δ^{13}C values were −24.5 and −24.6‰ for etiocholanolone and androsterone acetate, respectively. For week 19, the first post-T week, the values fell to −31.0 and −30.6‰. In the second post-T week (week 20), the values increased slightly to −30.6 and −29.9‰. In the subsequent weeks the δ^{13}C values gradually returned to the baseline. The mean difference between the δ^{13}C values before T and in week 19 was 6.5 and 6.1‰ for etiocholanolone and androsterone acetate, respectively. The T/E values returned to the baseline faster than the δ^{13}C values. By week 21 the T/E was less than the International Olympic Committee cutoff of 6, while the δ^{13}C values were still abnormally low.

Table 2. Between-assay precision. The δ^{13}C values of acetylated androsterone and etiocholanolone in the QC-Pos and QC-Neg were measured once/day on 25 days spanning 22 weeks

	QC-Neg δ^{13}C (‰)		QC-Pos δ^{13}C (‰)	
	Etio	Andro	Etio	Andro
Mean	−22.7	−22.5	−28.2	−28.4
SD	0.73	0.78	0.56	0.49
CV%	3.2	3.4	2.0	1.7
Range	2.8	2.9	2.9	2.8
Maximum	−21.6	−21.3	−27.2	−27.0
Minimum	−24.5	−24.2	−30.1	−29.8

Etio = Etiocholanolone acetate
Andro = Androsterone acetate

DISCUSSION

This GC/C/IRMS assay for urinary androsterone and etiocholanolone acetate produced complete separation of acetylated androsterone and etiocholanolone after a one-step extraction. The peak shapes were symmetrical, indicating the absence of co-eluting peaks, and virtually no other peaks were observed in the chromatograms. The analysis required only 2 mL of urine. Thirty samples can be processed in one batch, by one technician, in one day, including data analysis. Compared to our GC/C/IRMS diol assay,[6] this androsterone and etiocholanolone assay is faster, more robust, and provides lower CVs. The present assay is rapid and requires a sample volume of only 2 mL in part because androsterone and etiocholanone are present in urine at concentrations of 1000–4000 ng/mL whereas 5α- and 5β-androstane-3α,17β-diol are present at concentrations of 60–120 ng/mL.[6]

In this assay we measured the acetates of androsterone and etiocholanolone; however, other groups have investigated the measurement of underivatized urinary androsterone and etiocholanolone as indicators of the administration of T,[10] DHT and DHEA.[13] These assays required 2 to 20 mL urine, multiple extraction, and HPLC fractionation. Our studies of underivatized androsterone and etiocholanolone gave unsatisfactory results due to a peak that co-eluted with etiocholanolone. In addition, unlike in the present study, the δ^{13}C values of androsterone and etiocholanolone differed significantly.

Previously we measured δ^{13}C values of urinary T from 30–40 mL urine after extensive HPLC fractionation,[4,11] or from 10 mL urine using immunoaffinity chromatography and HPLC fractionation.[14] Later we determined δ^{13}C values for 5α- and 5β-androstane-3α,17β-diol using only 10 mL urine;[6] however, the assay required multiple extraction steps. Shackleton *et al.*[5] also measured the androstanediols; however, multiple extraction steps and one chemical oxidation step were needed.

The between-assay SDs for QC-Neg were 0.73 and 0.78‰ for etiocholanolone and androsterone acetate, while

Copyright © 2000 John Wiley & Sons, Ltd.

USADA 0784

the SDs for QC-Pos were smaller (0.56 and 0.49‰). The reason for the apparent difference between QC-Pos and QC-Neg is not known. As expected, the within-assay precision was also lower (range 0.15–0.46‰) for both steroids. No comparable data for between- or within-assay precision have been published.

The mean $\delta^{13}C$ values of the androsterone and etiocholanolone acetate in the QC-Pos and QC-Neg differed by less than 1% (0.2‰) (Table 2). This suggests that there was no difference between endogenous and exogenous T metabolism with respect to the proportions metabolized to androsterone and etiocholanolone. The androsterone and etiocholanolone acetate $\delta^{13}C$ values in multiple samples obtained after T administration were also virtually equal (Fig. 6), again suggesting the above. In contrast to these results, in our previous studies, the $\delta^{13}C$ values of the 5α- and 5β-androstane-3α,17β-diol metabolites of T (as diacetates) differed by up to 4‰.[6]

The absence of an endogenous reference compound in this method is a disadvantage; however, it is outweighed by the rapid throughput, low sample volume, and simplicity. The ideal screening assay would not miss potential positive cases and would register abnormal values if T had been administered. In the subject studied herein, the administration of T led to an easily detectable decline in androsterone and etiocholanolone acetate $\delta^{13}C$ values of about 6‰, and the values remained abnormal longer than the T/E remained >6.

The GC/C/IRMS method described here permits precise and accurate measurements of the $\delta^{13}C$ values of etiocholanolone and androsterone acetate from urine. This procedure was much faster and simpler than our previously reported methods for detecting T administration. The rapid sample throughput and low sample volume makes this assay suitable to serve as a screening assay for urine samples with T/E lower than 6 thereby increasing the ability of GC/C/IRMS methods to detect T administration. At this time we are ready to use this assay to screen urine samples with T/E both >6 and <6. If we found low $\delta^{13}C$ values, we would confirm the result with our diol assay.[6]

Acknowledgements

We thank K. Schramm for assistance in sample management and preparation. The research was supported by the National Collegiate Athletic Association, the National Football League and the United States Olympic Committee.

REFERENCES

1. Catlin DH, Murray TH. *JAMA* 1996; **276**: 231.
2. Catlin DH, Hatton CK, Starcevic SH. *Clin. Chem.* 1997; **43**: 1280.
3. Garle M, Ocka R, Palonek E, Bjorkhem I. *J. Chromatogr. B* 1996; **687**: 55.
4. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. *Rapid Commun. Mass Spectrom.* 1994; **8**: 304.
5. Shackleton CH, Phillips A, Chang T, Li Y. *Steroids* 1997; **62**: 379.
6. Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, Swerdloff RS, Pope HG, Hatton CK. *J. Chromatogr. B* 1999; **727**: 95.
7. Horning S, Geyer H, Machnic M, Schänzer W, Hilkert A, Oebelman J. In Recent Advances in Doping Analysis, vol. 4. Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U. (eds). Sport and Buch Strauss: Koln, Germany, 1996; 275.
8. Ueki M, Okano M. *Rapid Commun. Mass Spectrom.* 1999; **13**: 2237.
9. Shackleton CH, Roitman E, Phillips A, Chang T. *Steroids* 1997; **62**: 665.
10. Horning S, Geyer H, Gotzmann A, Schänzer W. In Recent Advances in Doping Analysis vol. 5. Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U. (eds). Sport and Buch Strauss: Koln, Germany, 1997; 135.
11. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG. *J. Mass Spectrom.* 1996; **31**: 169.
12. Catlin DH, Kammerer RC, Hatton CK, Sekera MH, Merdink JM. *Clin. Chem.* 1987; **38**: 319.
13. Flenker U, Horning S, Nolteernsting E, Geyer H, Schänzer W, In Recent Advances in Doping Analysis, vol. 6. Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U. (eds). Sport and Buch Strauss: Koln, Germany, 1998; 243.
14. Aguilera R, Becchi M, Grenot C, Casabianca H, Hatton CK. *J. Chromatogr. B* 1996; **687**: 43.

Copyright © 2000 John Wiley & Sons, Ltd.

USADA 0785

IRMS

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, xxx (2005) xxx–xxx
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Longitudinal profiling of urinary steroids by gas chromatography/combustion/isotope ratio mass spectrometry: Diet change may result in carbon isotopic variations

Christophe Saudan [a,*], Matthias Kamber [b], Giulia Barbati [c], Neil Robinson [a], Aurélien Desmarchelier [a], Patrice Mangin [a], Martial Saugy [a]

[a] *Laboratoire Suisse d'Analyse du Dopage, Institut Universitaire de Médecine Légale, Rue du Bugnon 21, 1005 Lausanne, Switzerland*
[b] *Swiss Federal Office of Sport, Magglingen, Switzerland*
[c] *Centro di Statistica Medica, AFaR, Ospedale Fatebenefratelli, Rome, Italy*

Received 13 June 2005; accepted 18 November 2005

Abstract

Longitudinal profiling of urinary steroids was investigated by using a gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) method. The carbon isotope ratio of three urinary testosterone (T) metabolites: androsterone, etiocholanolone, 5β-androstane-3α,17β-diol (5β-androstanediol) together with 16(5α)-androsten-3α-ol (androstenol) and 5β-pregnane-3α,20α-diol (5β-pregnanediol) were measured in urine samples collected from three top-level athletes over 2 years. Throughout the study, the subjects were living in Switzerland and were residing every year for a month or two in an African country. ^{13}C-enrichment larger than 2.5‰ was observed for one subject after a 2-month stay in Africa. Our findings reveal that ^{13}C-enrichment caused by a diet change might be reduced if the stay in Africa was shorter or if the urine sample was not collected within the days after return to Switzerland. The steroids of interest in each sample did not show significant isotopic fractionation that could lead to false positive results in anti-doping testing. In contrast to the results obtained with the carbon isotopic ratio, profiling of urinary testosterone/epitestosterone (T/E) ratios was found to be unaffected by a diet change.
© 2005 Published by Elsevier B.V.

Keywords: Steroids; Diet; Isotope ratio mass spectrometry (IRMS); Doping control

1. Introduction

Endogenous steroids are produced from cholesterol in the body. Cholesterol is derived from an average of a wide variety of feed vegetal and animal precursors or synthesized from precursors of feed origin. In plant tissue, the main source of variation in $^{13}C/^{12}C$ isotopic ratio (expressed in $\delta^{13}C$-value) is derived from the different photosynthetic pathways for carbon dioxide fixation. Plants incorporate carbon dioxide photosynthetically by three different mechanisms: the Calvin cycle (C_3) pathway, the Hatch-Slack (C_4) pathway and the Crassulacean acid metabolism (CAM) pathway. The C_3 pathway results in a large change in the carbon isotope proportions relative to atmospheric carbon dioxide and hence discriminates more strongly against the heavier isotope ^{13}C compared to the C_4 pathway. Main representatives of C_3 group are wheat, rice, potato, barley, grape, oats and sugar beet, whereas maize, sugar cane, millet and pineapple are the important species of the C_4 group. The $\delta^{13}C$-values of C_3 and C_4 plants are ranging from −35 to −22‰ and from −20 to −8‰, respectively [1].

The difference in the ^{13}C enrichment of food products in the diet and even in the food chain is caused by different contribution of naturally ^{13}C-enriched constituents. Because common food ingredients are maize, millet and sugar cane (C_4 plants) in certain areas of Africa, it is expected that the basic ^{13}C enrichment of the body store will be high for local populations [2]. It is known that urine samples collected from a country like Kenya have a higher content of ^{13}C in steroids than western or oceanian countries [3,4].

Although carbon isotope dynamic studies of urinary steroids in relation with diet changes have been shown to produce significant variations in cattle [5,6], little is known on exchange

* Corresponding author. Tel.: +41 213147106.
E-mail address: christophe.saudan@chuv.ch (C. Saudan).

1570-0232/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2005.11.029

rates of ^{12}C and ^{13}C during steroid biosynthesis in humans. A GC/CIRMS method is used to investigate longitudinal variations over a 2-year period of carbon isotopic ratio ($^{13}C/^{12}C$) of urinary steroids in selected top-level Caucasian male athletes and also assess on their profiles, the possible influence of environmental/diet changes induced by intercontinental traveling with variable residence time periods. These data should demonstrate the use of IRMS analysis for longitudinal profiling and provide insights into physiological variations of steroid carbon isotopes, also with the aim of avoiding misinterpretation in isotope ratio mass spectrometry (IRMS) anti-doping testing.

2. Experimental

2.1. Subjects and experimental protocol

Three top-level Caucasian male athletes (runners) aged between 28 and 30 years participated in a 2-year longitudinal study. This study is a part of the project "Top Level Sport Without Doping" of the Swiss Federal Office for Sport (FOSPO) with the aim of promoting doping-free sport and protecting the right of athletes to compete in a fair and ethical sport environment. The participants gave their written informed consent and accepted to produce urine samples for both announced and unannounced testing. Throughout this 2-year study, the subjects were residing from 1 to 2 months a year in either Kenya or South Africa, and were living in Switzerland for the rest of the time or were in competition in a European country for 2-week maximum period. Although no detailed diet report was requested, the subjects declared that they had fully adapted their diet to local food, with the two main changes being an increase in maize consumption and substituting cane sugar for beet sugar. In addition, none of the athletes consumed food supplement or prohormones during the entire study. Urine samples were divided into 20 ml flasks and stored without additives at −20 °C until analysis.

2.2. Quantitative analysis of urinary steroids

Quantitative analyses of urinary testosterone (4-androsten-17β-ol-3-one) and epitestosterone (4-androsten-17α-ol-3-one) glucuronides were performed using similar procedures of a previously reported method [7]. The urine sample (2.5 ml) was spiked with 20 μl of an internal standard (17α-methyltestosterone, 0.020 mg/ml in methanol) and was then applied onto a 500 mg C_{18} solid phase column (JT Baker, Phillipsburg, NJ, USA) previously conditioned with 5 ml of methanol and 5 ml of water. Elution was carried out three times with 1 ml of methanol and the solvent was evaporated to dryness under a stream of nitrogen at 40 °C (Turbo Vap LV evaporator, Zymark, Hopkinton, MA, USA). To hydrolyze the glucoconjugated steroids, the residue was dissolved in 1 ml of 0.2 M phosphate buffer (pH 7.0) and 50 μl of β-glucuronidase from *Escherichia coli* in a 50% glycerol solution (pH 6.5, 140 U/ml at 37 °C) was added as supplied (Roche Diagnostics GmbH, Manheim, Germany). The hydrolysis was completed in 1 h at 50 °C or at 37 °C overnight in a thermostated water bath. After addition of about 200 mg solid carbonate buffer (Na_2CO_3–$NaHCO_3$, 1:10 by weight), the sample was extracted with 5 ml of *tert*-butylmethylether (TBME) with shaking for 10 min. After centrifugation (2500 × *g* for 5 min), the organic phase was collected and dried with solid Na_2SO_4. The derivatization was carried out by addition of 50 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA)/NH_4I/ethanethiol (1000/2/3; v/w/v). The reaction mixture was heated in a stopped vial at 60 °C for 20 min. The samples (2 μl) were injected at 270 °C with a 10:1 split on a Hewlett-Packard 6890 Serie II Plus chromatograph (HP Analytical Division, Waldbronn, Germany) equipped with a HP 7683 auto-sampler and coupled with a HP 5973 mass selective detector (MSD). GC separation was achieved on HP-1 column (17 m × 0.2 mm i.d., 0.11 μm film thickness) from J&W Scientific (Folsom, CA, USA) operated with a helium inlet pressure of 20 psi. The oven temperature was increased from 181 to 230 °C at 3 °C/min, and then to 310 °C at 40 °C/min and held for 4 min. The electronic beam energy was set at 70 eV in the electronic impact (EI) mode.

The analyses were performed in single ion monitoring mode (SIM) with $m/z = 432$ for both testosterone and epitestosterone. For each substance, a six-point calibration curve was established ($R^2 > 0.996$) using available reference material (Steraloids Inc., Newport, RI, USA) in the 5–250 ng/ml concentration range for testosterone and epitestosterone.

2.3. GC/C/IRMS analysis of urine samples

The symbol δ is the standard notation for expressing carbon isotope ratios $^{13}C/^{12}C$. It is defined as parts per thousand deviation of isotopic compositions from that of Pee Dee Belemnite (PDB), and is calculated according to:

$$\delta^{13}C\,(‰) = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 1000$$

Urinary androstenol (16(5α)-androsten-3α-ol), 5β-pregnanediol (5β-pregnane-3α,20α-diol), androsterone (5α-androstan-3α-ol-17-one), etiocholanolone (5β-androstan-3α-ol-17-one) and 5β-androstanediol (5β-androstane-3α,17β-diol) were extracted in two different fractions following a previously described method for IRMS analysis [8]. A Delta^{Plus} XL IRMS system (Thermo Finnigan MAT, Bremen, Germany) coupled to an Agilent 6890A Gas Chromatograph (HP Analytical Division, Waldbronn, Germany) via a Finnigan GC Combustion III interface (Thermo Finnigan MAT, Bremen, Germany) and a CTC Analytics CombiPal auto-sampler (CTC Analytics AG, Zwingen, Switzerland) were used for separation and online combustion of the steroid metabolites. Chromatographic separations were achieved on a HP cross-linked 50% phenylmethylsiloxane fused silica capillary column (30 m × 0.25 mm i.d., 0.15 μm film thickness) from J&W Scientific (Folsom, CA, USA). The carrier gas was helium with a constant flow of 1.0 ml/min and initial pressure of 13.6 psi. The injection volume was 2 μl in splitless mode at 280 °C. The combustion and reduction oven temperatures were set to 940 and 600 °C, respectively. For the analysis of the androsterone and etio-

cholanolone acetates, the oven temperature was increased from 80 °C (1 min) to 270 °C (8.3 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. For the analysis of 5β-androstanediol, 5β-pregnanediol and androstenol acetates, the oven temperature was increased from 80 °C (1 min) to 270 °C (11.5 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. All urine samples of each athlete have been processed by batch to obtain a sufficient precision on the carbon isotope ratios (S.D. ~0.3‰). Negative shifts of the δ^{13}C-value due to the formation of acetate were corrected [8].

2.4. Statistical analysis

Statistical analysis was performed using the SPPS 12.0 package for Windows (SPPS Inc., Chicago, USA). The normality of the distributions was assessed with the Kolmogorov–Smirnov test. Statistical significance for the carbon isotopic values of the first urine sample collected from each subject after residence periods in Kenya and South Africa with respect to the other samples was assessed with the Wilcoxon signed-ranks test. The significance level was set at $p < 0.05$.

3. Results and discussion

The 2-year longitudinal profile of urinary isotopic values (expressed as δ^{13}C-value) of measured steroids is depicted for each subject in Fig. 1, together with their residence periods in Africa. Using Wilcoxon signed-ranks test, significant changes ($p < 0.05$) are observed for the mean carbon isotopic values of the first urine sample collected from subject S1 after the residence periods in Kenya and South Africa. While mean carbon isotopic values of the urine sample of subject S2 directly collected after returning from the second period in South Africa are significantly different from the values of the other samples, the longitudinal profile of subject S3 reveals no significant variation throughout the 2-year study. After residence in African countries, the observed variations versus less negative delta values, i.e. versus enrichment in ^{13}C, may mainly be attributed to a change in diet [2]. Significant differences ($\Delta\delta^{13}$C-value > 3‰) are observed when the mean carbon isotopic values of urinary androsterone and etiocholanolone in Kenyan [3] and Swiss [4] populations are compared.

Our results tend to show a dynamic ^{13}C-enrichment upon diet change with significant inter-individual variations following time of residence and collection time interval of the urine sample after returning to Switzerland. Urine samples of subject S3 collected 2 and 1 months after the first and second 5-week residence in Kenya, respectively, do not show significant ^{13}C-enrichment, whereas a variation of about 1‰ is observed for the urine sample collected 2 weeks after returning to Switzerland (subject S2, Fig. 1). In contrast to subjects S1 and S2, the natural longitudinal variation in the carbon isotopic ratios of urinary steroids is normally distributed for subject S3, and the standard deviations for the steroids of interest are ranging from 0.21 to 0.38‰. The profile of subject S1 is characterized by a large variation ($\Delta\delta^{13}$C-values > 2.5‰) for the urine sample col-

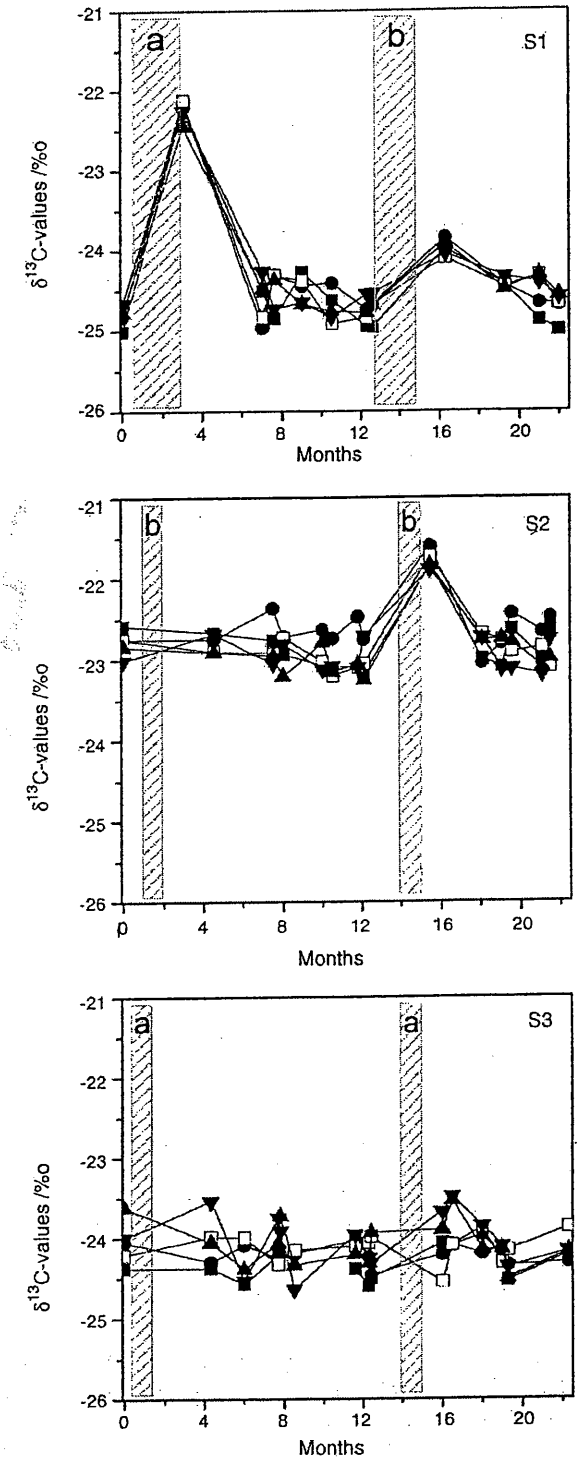


Fig. 1. Two-year longitudinal profiles of urinary δ^{13}C-values of androsterone (▲), etiocholanolone (▼), 5β-pregnanediol (●), 5β-androstanediol (■) and androstenol (□) for athletes S1, S2 and S3. The striped rectangles denote the residence periods in either Kenya (a) or South Africa (b). Residence time of subject S1 in Africa was of 10 and 8 weeks for the first and second year, respectively. Subjects S2 and S3 resided for 5 weeks per year in South Africa and Kenya, respectively. Collection time interval of the first urine after the first residence in Africa was of 1, 8 and 9 weeks for subjects S1, S2 and S3, respectively. After the second residence in Africa, the urine samples were collected at the earliest 6, 2 and 4 weeks for subjects S1, S2 and S3, respectively.

lected 1 week after returning from Kenya and a much smaller variation for the urine sample collected 6 weeks after the stay in South Africa. Nevertheless, it is not possible from our data to anticipate a larger ^{13}C-enrichment in subject S1 if (1) he would have resided for a longer period of time in Kenya and (2) the urine sample would have been collected the day after returning to Switzerland.

The selected urinary steroids are all derived from cholesterol, but are produced along different metabolic pathways. Therefore, significant differences in ^{13}C-enrichment of these steroids induced by different carbon exchanges could take place within a short period of time after diet changes with ^{13}C-enriched ingredients. For the first urine samples collected after intercontinental stays of subjects S1 and S2, differences less than 0.4‰ are found between the steroids of interest. Considering the within-assay precision on IRMS measurements, these results show that similar carbon redistribution along the different metabolic pathways was reached, either with enrichment in ^{13}C or with a return to basal ^{13}C. For subject S3, we obtain larger differences ($\Delta\delta^{13}C$-values up to 0.9‰) for the first urine sample collected after both 1-month stays in Kenya.

The International Olympic Committee (IOC) Medical Commission banned the use of testosterone in 1982 and stated that a urinary T/E ratio above 6 was a sufficient proof of testosterone abuse. After while, it became apparent that certain individuals had T/E ratios naturally elevated above 6 [9], the IOC stated that a follow-up was needed for T/E ratios above this value to prove physiological or pathological circumstances. In August 2004, a technical document from the World Anti-Doping Agency (WADA) was adopted with the need of submitting the sample to IRMS analysis for determination of the $^{13}C/^{12}C$ ratio of selected steroids, if the urinary T/E ratio value is equal or greater than 4 as well as for altered steroid profiles with testosterone metabolites and DHEA concentration greater than fixed cut-off concentrations [10]. If IRMS analysis does not readily indicate exogenous administration of steroids for urinary samples with T/E ratio ≥ 4, the result is reported as inconclusive and further longitudinal studies have to be performed.

As exogenous testosterone or precursors contain less ^{13}C than their endogenous homologues, it is expected that urinary steroids with a more negative $^{13}C/^{12}C$ ratio originate from pharmaceutical source [11]. According to the technical document of the WADA Laboratory Committee, a result would be reported as consistent with the administration of a steroid when the $^{13}C/^{12}C$ value measured for the metabolites differs significantly, i.e. by 3.0‰ or more from that of the urinary reference steroid chosen [10]. We observe that carbon isotopic differences between testosterone metabolites (androsterone, etiocholanolone and 5β-androstanediol) and endogenous references (androstenol and 5β-pregnanediol) are less than 0.8, 0.7 and 0.9‰ for subjects S1, S2 and S3, respectively. Therefore, these results do not indicate a doping with testosterone or a precursor, according to August 2004 WADA criteria.

For the urinary T/E–time profiles of each subject, it was found that the T/E ratios are normally distributed. All subjects have a remarkably stable T/E ratio throughout the study with coefficients of variation less than 15%. The mean T/E ratio was 1.37 (range: 1.09–1.56, $n=9$), 0.96 (range: 0.84–1.14, $n=10$) and 1.18 (range: 0.80–1.36, $n=10$) for subjects S1, S2 and S3, respectively. These results are in accordance with longitudinal T/E ratio profiles of drug-free males [12]. Nevertheless, it should be mentioned that these T/E–time profiles together with longitudinal carbon isotopic variations may point out a doping with endogenous steroids, but are likely not sensitive enough to detect a doping with anabolic steroids of synthetic origin [13].

In conclusion, our findings have shown that the stay, the length of stay in an intercontinental country and time collection of urine samples might be factors affecting the ^{13}C content of steroids, but do not induce significant isotopic fractionations that could lead to false positive results in anti-doping analysis. Nevertheless, this study is a starting point and further investigations should be performed with a higher number of male and female subjects, together with different residence times and urine sample collections to describe a general kinetic model of carbon enrichment or depletion in steroids. IRMS has been claimed to be the definitive solution for interpretation of abnormal endogenous profiles in drug testing. However, when drug-testing authorities interpret IRMS results, they should have detailed information about residencies and travels of the athlete (whereabouts) as diet might influence the $^{13}C/^{12}C$ values of urinary steroids.

Acknowledgements

We thank Dr. Simone Bader and Sabine Dominé for their technical assistance. This research was entirely supported by the Swiss Federal Office for Sport (FOSPO).

References

[1] I.T. Platzner, K. Habfast, A.J. Walder, A. Goetz, in: J.D. Winefordner (Ed.), Modern Isotope Ratio Mass Spectrometry, J Wiley & Sons, New York, 1997, p. 403.
[2] A.A. Tanis, T. Rietveld, J.W.O. Van Den Berg, J.L. Darcos Wattimena, G.R. Swart, Nutrition 16 (2000) 6.
[3] A. Cawley, J. Rogerson, K. Rahman, G. Trout, R. Kazlauskas, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Sport und Buch Strauß, Köln, 2003, p. 183.
[4] A. Maître, C. Saudan, P. Mangin, M. Saugy, J. Anal. Toxicol. 28 (2004) 426.
[5] V. Ferchaud, B. Le Bizec, F. Monteau, F. Andre, Rapid Commun. Mass Spectrom. 14 (2000) 652.
[6] G. Balizs, A. Jainz, P. Horvatovich, J. Chromatogr. A 1067 (2005) 323.
[7] S.H. Peng, J. Segura, M. Farré, J.C. González, X. de la Torre, Steroids 67 (2002) 39.
[8] C. Saudan, N. Baume, P. Mangin, M. Saugy, J. Chromatogr. B 810 (2004) 157.
[9] E. Raynaud, M. Audran, J.F. Brun, C. Fedou, J.L. Chanal, A. Orsetti, Lancet 340 (1992) 1468.
[10] WADA Technical Document: Reporting and evaluation guidance for testosterone, epitestosterone, T/E ratio and other endogenous steroids, 2004. http://www.wada-ama.org/.
[11] M. Ueki, M. Okano, Rapid Commun. Mass Spectrom. 13 (1999) 2237.
[12] D.H. Catlin, C.K. Hatton, S.H. Starcevic, Clin. Chem. 43 (1997) 1280.
[13] D.H. van de Kerhof, D. de Boer, J.H.H. Thijssen, R.A.A. Maes, J. Anal. Toxicol. 24 (2000) 102.

REVIEWED BY DATE 4/06
ACTION:
☐ FORWARD TO:
☐ FOLLOWUP
☒ FILE: TRMS
M UC Y JOURNAL OF Y+7 (P)
CHROMATOGRAPHY B

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 810 (2004) 157–164
www.elsevier.com/locate/chromb

Urinary analysis of 16(5α)-androsten-3α-ol by gas chromatography/combustion/isotope ratio mass spectrometry: implications in anti-doping analysis

Christophe Saudan*, Norbert Baume, Patrice Mangin, Martial Saugy

Laboratoire Suisse d'Analyse du Dopage, Institut Universitaire de Médecine Légale, Rue du Bugnon 21, 1005 Lausanne, Switzerland

Received 11 June 2004; accepted 27 July 2004
Available online 26 August 2004

Abstract

We present a method for the analysis of urinary 16(5α)-androsten-3α-ol together with 5β-pregnane-3α,20α-diol and four testosterone metabolites: androsterone (Andro), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αA), 5β-androstane-3α,17β-diol (5βA) by means of gas chromatography/combustion/isotopic ratio mass spectrometry (GC/C/IRMS). The within-assay and between-assay precision S.D.s of the investigated steroids were lower than 0.3 and 0.6‰, respectively. A comparative study on a population composed of 20 subjects has shown that the differences of the intra-individual δ^{13}C-values for 16(5α)-androsten-3α-ol and 5β-pregnane-3α,20α-diol are less than 0.9‰. Thereafter, the method has been applied in the frame of an excretion study following oral ingestion of 50 mg DHEA initially and oral ingestion of 50 mg pregnenolone 48 h later. Our findings show that administration of DHEA does not affect the isotopic ratio values of 16(5α)-androsten-3α-ol and 5β-pregnane-3α,20α-diol, whereas the isotopic ratio values of 5β-pregnane-3α,20α-diol vary by more 5‰ upon ingestion of pregnenolone. We have observed δ^{13}C-value changes lower than 1‰ for 16(5α)-androsten-3α-ol, though pregnenolone is a precursor of the 16-ene steroids. In contrast to 5β-pregnane-3α,20α-diol, the 16-ene steroid may be used as an endogenous reference compound when pregnenolone is administered.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Androstenol; Pregnenolone; Isotope ratio mass spectrometry (IRMS); Doping control

1. Introduction

16(5α)-Androsten-3α-ol (Andro) is excreted in human urine with mean values of 1 mg/24 h and 0.3 mg/24 h by men and women, respectively. The amounts excreted by prepubertal children and by elderly men and post-menopausal women are considerably lower than these mean values [1]. The precise biosynthesis of androstenol has been subject for debate. Recent studies revealed that androstadienol (5,16-androstadien-3β-ol) is synthesized from pregnenolone in a single step (Fig. 1) by a 16-ene-synthase enzyme system in human testicular homogenates [2]. Very recently, it has been demonstrated that P450c17 possesses a 16-ene-synthase activity capable to transform pregnenolone into androstadienol without the formation of a precursor [3]. Further transformation of androstadienol into androstenol involves the classic enzymes of the steroidogenic pathway [4]. Although it is known that androstenol has a pheromonal activity in pigs, the role of this steroid in humans is still ill-defined [1]. It has been suggested recently that menstrual synchrony is related to the ability to perceive the odour emitted by androstenol [5]. A study reports also the ability of androstenol to bind and deactivate the nuclear receptor CAR-β [6].

Isotope ratio mass spectrometry (IRMS) allows measurements of slight differences in the carbon isotope ratio (^{13}C/^{12}C) of the exogenous and endogenous steroids. Synthetic steroids are generally produced from precursors derived from plants with low ^{13}C content, whereas the ^{13}C and ^{12}C content in the natural endogenous form depends on the

* Corresponding author. Tel.: +41 213 147106; fax: +41 213 147333.
E-mail address: csaudan@hospvd.ch (C. Saudan).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.07.029

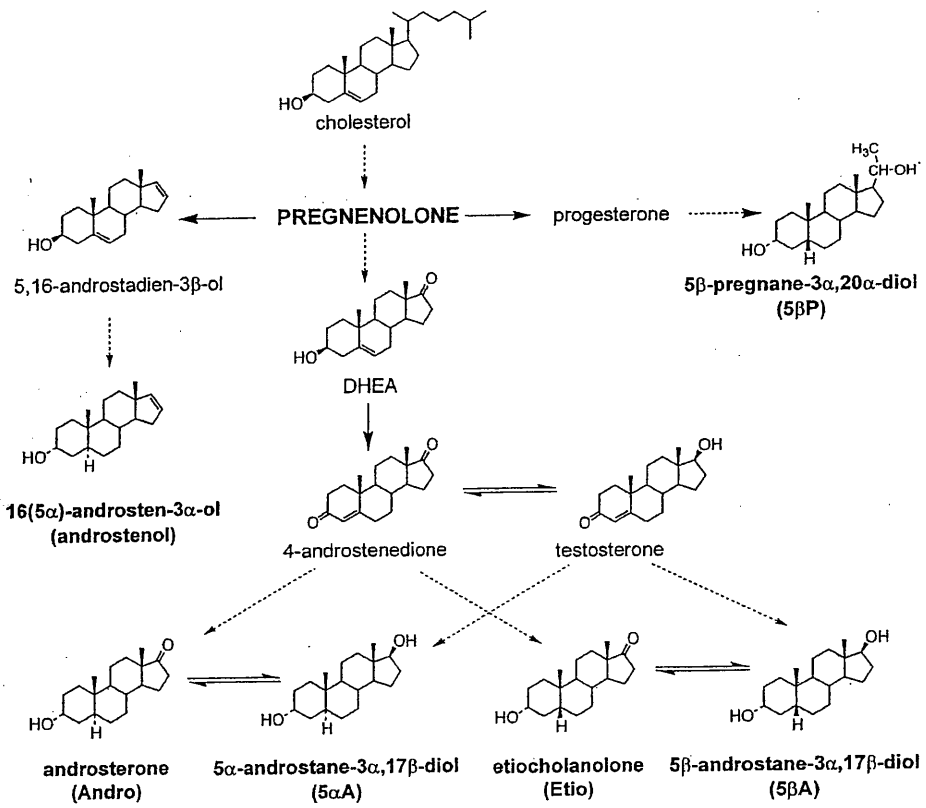


Fig. 1. Simplified metabolic pathways of pregnenolone.

isotopic carbon composition of the food diet and is influenced by additional effects of human biological processing [7]. Such differentiation between synthetic and endogenous origins has been reported for misuse in sport of steroids such as testosterone [7–9], DHEA [10], epitestosterone [11], DHT [12,13], nandrolone [14] and corticosteroids [15]. For IRMS measurements, where the inter-individual variability and instrument calibration may significantly influence the results, it is recommended to relate the difference of the steroids isotopic value to that of an endogenous reference compound [16]. For that purpose, cholesterol and 5β-pregnane-3α,20α-diol (5βP) are usually used as an endogenous reference compound in IRMS measurements because the isotopic values should not be affected by the administration of exogenous steroids such as testosterone [7,8], DHEA [10], DHT [12] and androstenedione [17]. However, the origin of urinary cholesterol is not well understood [7] and it has been also demonstrated that the use of 5β-pregnane-3α,20α-diol as endogenous reference compound is not longer applicable when pregnenolone is ingested [18].

In this paper, we present a method for the analysis of urinary androstenol together with 5β-pregnane-3α,20α-diol (5βP) and four testosterone metabolites: androsterone, etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αA), 5β-androstane-3α,17β-diol (5βA) by means of gas chromatography/combustion/isotopic ratio mass spectrometry (GC/C/IRMS). We show that androstenol may be used as an endogenous reference compound and to what extent the administration of pregnenolone will affect the isotopic ratio values of both androstenol and 5β-pregnane-3α,20α-diol. Indeed, pregnenolone is a precursor of 5β-pregnane-3α,20α-diol and androstenol (Fig. 1) and is a freely available compound in the United State, on internet and may be found in nutritional supplements with other pro-hormones. It is supposed that pregnenolone has supporting benefits for memory, cognitive function, joint regeneration, skin conditions and firmness of skin.

2. Experimental

2.1. Chemicals

All solvents and reagents of analytical grade purity were purchased from Fluka (Buchs, Switzerland). 5α-Androstan-3β-ol acetate and 5β-pregnane-3α,20α-diol were obtained from Steraloids Inc. (Newport, RI, USA). 5α-Androstane-3α,17β-diol, 5β-androstane-3α,17β-diol and 16(5α)-androsten-3α-ol were supplied by Sigma (St. Louis, MO, USA). Etiocholanolone and androsterone were purchased from NARL (Pymble, Australia). Bakerbond spe™ 500 mg or 1000 mg octadecyl C_{18} disposable extraction columns were obtained from JT Baker (Phillipsburg, NJ, USA). β-Glucuronidase from *Escherichia coli* in a 50% glyc-

USADA 0791

erol solution (pH 6.5, 140 U/ml at 37 °C) was supplied by Roche Diagnostics GmbH (Manheim, Germany). The reference carbon dioxide gas was purchased from Carbagaz (Domdidier, Switzerland). The mixture of three alkanes, C_{15} (*n*-pentadecane), C_{20} (*n*-eicosane) and C_{25} (*n*-pentacosane) was supplied by Chiron AS (Trondheim, Norway).

2.2. Dosage and collection of urine samples

Two 25 mg DHEA capsules (Schiff®, Salt Lake City, UT, USA) were self-administered orally initially and one 50 mg pregnenolone capsule (Metabolic Response Modifier™, San Clemente, CA, USA) was self-administered orally 48 h later by a Caucasian healthy male volunteer. Baseline urine samples were obtained before initial administration, and subsequent spot urine samples were collected over a period of 155 h after the first capsule administration. Reference urine samples were collected from 20 Caucasian male and female subjects living in Switzerland. The positive quality control urine (QC-pos) was obtained from an excretion study after ingestion of a 40 mg testosterone undecanoate capsule (Panteston®, Organon SA, France) whereas the negative quality control urine (QC-neg) was obtained from a healthy subject. Each urine was aliquoted into 20 ml cryogenic tubes and stored without additives at −20 °C until analysis.

2.3. Sample preparation

The urine sample (10 ml) was centrifugated in glass tubes at 2500 rpm for 5 min and was applied onto a C_{18} column (500 mg). The conjugated steroids were eluted with methanol (8 ml) and the eluate evaporated to dryness. To hydrolyze the conjugated steroids, the residue was dissolved in 1 ml of 0.2 M phosphate buffer (pH 7.0) and 50 μl of β-glucuronidase was added as supplied. After incubation at 50 °C during 1 h (or at 37 °C overnight) in a thermostated water bath, the deconjugated steroids were extracted on C_{18} column (500 mg) by washing with 6 ml acetonitrile:H_2O (20:80 (v/v)) and subsequently by 6 ml acetonitrile:H_2O (35:65 (v/v)) and finally eluted with 12 ml acetonitrile. The eluate was evaporated to dryness under a nitrogen stream (Turbo Vap LV evaporator, Zymark, Hopkinton, MA, USA) and then the residue was dried over phosphorus pentoxide for 20 min. Acetylation of the extract was carried out in 50 μl of pyridine and 50 μl of acetic anhydride at 60 °C for 1 h incubation. The reaction medium was evaporated to dryness and subsequently the residue was redissolved in 3 ml of acetonitrile:H_2O (50:50 (v/v)). The solution was applied onto a C_{18} column (1000 mg). The fraction F1 containing androsterone and etiocholanolone was obtained by elution with 15 ml acetonitrile:H_2O (75:25 (v/v)) after washing with 12 ml of acetonitrile:H_2O (50:50 (v/v)). Finally, the fraction F2 with the androstandiols, the pregnanediol and the androstenol was obtained by eluting with 15 ml of acetonitrile. The derivatized steroids of fractions F1 and F2 were spiked with 80 and 10 μl of an internal standard (IS, 5α-androstan-3β-ol acetate, 0.20 mg/ml in methanol), respectively. Then, both fractions F1 and F2 were evaporated to dryness and finally dissolved in 500 and 40 μl of cyclohexane, respectively.

2.4. Determination of androstenol and 5βP concentrations

Urinary concentration of androstenol and 5βP were quantified in the reference urine samples collected from 20 Caucasian male and female subjects. Quantitative analyses were performed on a Hewlett-Packard 5890 Serie II Plus chromatograph (HP Analytical Division, Waldbronn, Germany) equipped with a HP 7673 auto-sampler and coupled with a HP 5971 mass selective detector (MSD). GC separation was achieved on a ZB-5 column (15 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA) operated with a helium inlet pressure of 103 kPa. The oven temperature was increased from 80 (1 min) to 270 °C (6.0 min) at 15 °C/min, then to 300 °C at 15 °C/min. Injections of 1 μl-samples from fraction F2 were made at 280 °C in the splitless mode. The analyses were performed in single ion monitoring mode (SIM) with ions $m/z = 316$ for androstenol acetate, $m/z = 344$ for 5βP acetate and $m/z = 204, 258$ for 5α-androstan-3β-ol acetate (IS). Regression curves with 30, 60, 120, 300, 600 ng/μl androstenol ($R^2 = 0.994$) and 5β-P ($R^2 = 0.993$) solutions were used for the quantification of both steroids concentrations. EI mass spectra were recorded by continuous scanning in the mass range 50–450 at an ionization potential of 70 eV.

2.5. GC/C/IRMS analysis

The carbon isotope measurements were performed on a Delta^{Plus} IRMS system (Thermo Finnigan MAT, Bremen, Germany) coupled to an Agilent 6890A gas chromatograph (HP Analytical Division, Waldbronn, Germany) via a Finnigan GC Combustion III interface (Thermo Finnigan MAT, Bremen, Germany) and a CTC Analytics CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland). The mass spectrometer consisted of an electron impact source held at 3 kV acceleration voltage for CO_2 gas. Chromatographic separations were achieved on a HP cross-linked 50% phenylmethylsiloxane fused silica capillary column (30 m × 0.25 mm i.d., 0.15 μm film thickness) from J&W Scientific. The injector temperature was set to 280 °C. The combustion and reduction oven temperatures were set to 940 and 600 °C, respectively. Reference carbon dioxide gas pulses (20 s durations) were introduced at four different times during the course of the chromatographic separation. For the calibration of the reference gas with the alkane mixture of known $\delta^{13}C$-values, the oven temperature was increased from 80 (1 min) to 270 °C (7.0 min) at 15 °C/min. For the analysis of the androsterone and etiocholanolone acetates (fraction F1), the oven temperature was increased from 80 (1 min) to 270 °C (8.3 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. For the analysis

USADA 0792

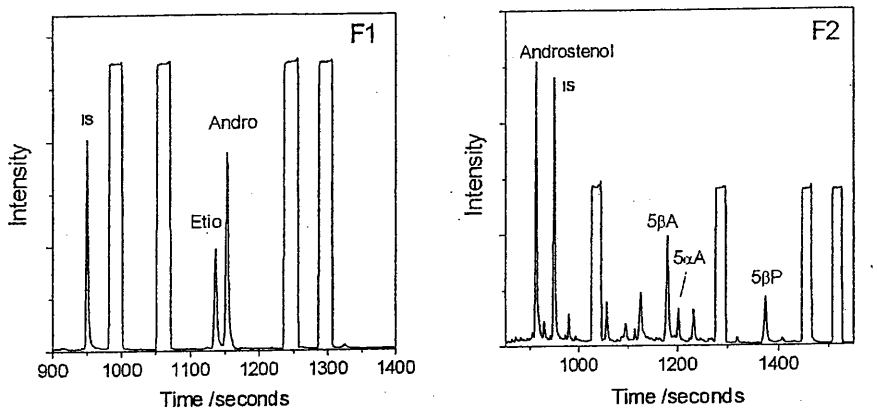


Fig. 2. Typical GC-C-IRMS *m/z*44 mass chromatograms of fractions F1 (androsterone and etiocholanolone acetates) and F2 (5αA, 5β5A, 5β5P and androstenol acetates) spiked with an internal standard (IS, 5α-androstan-3β-ol acetate). The square-topped peaks represent pulses of CO_2 reference gas.

of the fraction F2 containing the androstandiols, pregnanediol and androstenol acetates, the oven temperature was increased from 80 (1 min) to 270 °C (11.5 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. The volume of injection was 1–2 μl, depending on the concentrations of the compounds of interest. The extracts were injected once in splitless mode.

The symbol δ is the standard notation for expressing carbon isotope ratios. It is defined as parts per thousand deviation of isotopic compositions from that of Pee Dee Belemnite (PDB), and is calculated according to:

$$\delta^{13}C/\text{‰} = \frac{(^{13}C/^{12}C)_{\text{sample}} - (^{13}C/^{12}C)_{\text{standard}}}{(^{13}C/^{12}C)_{\text{standard}}} \times 1000$$

Negative shifts of the δ^{13}C-value due to the formation of an acetate were corrected as follows:

$$D_{OH} = D_{OAC} + 2m\frac{D_{OAC} - D_{AC}}{n}$$

where D_{OH} is δ^{13}C-value for the underivatized steroids, D_{OAC} the δ^{13}C-value for the acetylated steroids, D_{AC} the δ^{13}C-value for the acetylating reagent, n the number of carbon atom in a molecule and m the number of hydroxyl groups to be acetylated.

All subsequent δ^{13}C-values have been corrected for this negative shift. The D_{AC} value of our acetylating reagent was determined to be −48.8‰ according to a published procedure [19].

3. Results and discussion

3.1. Extraction method, isotope measurements, quality control and precision

The reference carbon dioxide gas which is pulsed several times during the course of the chromatographic separation is calibrated by a mixture of three alkanes, C_{15} (*n*-pentadecane), C_{20} (*n*-eicosane) and C_{25} (*n*-pentacosane) from Chiron AS with δ^{13}C-values of −30.22, −33.06 and −28.21‰, respectively.

The extraction method allows the separation of androsterone and etiocholanolone acetates from the 5α-androstane-3α,17β-diol (5αA), 5β-androstane-3α,17β-diol, 5β-pregnane-3α,20α-diol and androstenol acetates on the C_{18} column by eluting with different ratio mixture of acetonitrile/H_2O [18]. Before the GC/C/IRMS analysis, an internal standard (5α-androstan-3β-ol acetate) is spiked in both fractions. Fig. 2 shows an example of GC/C/IRMS chromatograms of *m/z* 44 for fractions F1 and F2. The chromatograms show symmetrical peaks for the compounds of interest, no tailing, and no evidence of co-eluting compounds. The peaks of GC/C/IRMS chromatograms were attributed to the assigned compounds after comparison of their retention time and mass spectra by GC/MS with those of authentic samples of the compounds of interest.

Three urines (6 ml) of a 3-year-old boy spiked with a 10 μl solution of the steroids of interest (1 mg/ml in methanol) were used to determine the recovery yields and the possible isotopic discrimination of the steroids during the separation and analysis procedure. We have obtained recovery yields of 95, 90, 92, 88, 80 and 58% for Andro, Etio, 5αA, 5βA, 5βP and androstenol, respectively. The isotopic ratio value of the spiked androstenol in urine ($\delta^{13}C_{\text{androstenol}} = -29.92$‰) was very similar to the standard value ($\delta^{13}C_{\text{androstenol}} = -30.40$‰). For the other steroids, we have obtained $\Delta\delta^{13}$C-values lower than 0.5‰ between the δ^{13}C-values of the spiked substances in the urine and the standards. Although the recovery yields of the steroids of interest are not quantitative, our findings tend to show that there is no significant isotopic discrimination during the separation and analysis procedure.

Each batch includes positive (QC-Pos) and negative (QC-Neg) quality controls and the measurements are performed in the linear range of the IRMS. The IRMS response of the steroids of interest was found linear for injected quantities

USADA 0793

Table 1
Within-assay precision for etiocholanolone, androsterone, 5βA, 5αA, 5βP and androstenol ($n = 5$)

	QC-Neg						QC-Pos					
	Etio	Andro	5βA	5αA	5βP	Androstenol	Etio	Andro	5βA	5αA	5βP	Androstenol
Mean	−24.12	−23.98	−24.37	−24.17	−23.57	−24.13	−27.57	−27.54	−28.20	−28.14	−23.61	−23.98
S.D.	0.19	0.20	0.20	0.22	0.29	0.11	0.16	0.17	0.19	0.25	0.29	0.24
CV (%)	0.8	0.9	0.8	0.9	1.2	0.4	0.6	0.6	0.7	0.9	1.2	1.0
Minimum (‰)	−24.39	−24.42	−24.47	−24.47	−23.93	−24.31	−27.83	−27.91	−28.46	−28.63	−24.02	−24.33
Maximum (‰)	−23.82	−23.82	−23.93	−23.86	−23.08	−23.95	−27.35	−27.42	−27.83	−27.78	−23.12	−23.76

Table 2
Between-assay precision for etiocholanolone, androsterone, 5βA, 5αA, 5βP and androstenol ($n = 16$)

	QC-Neg						QC-Pos					
	Etio	Andro	5βA	5αA	5βP	Androstenol	Etio	Andro	5βA	5αA	5βP	Androstenol
Mean	−24.23	−24.08	−24.37	−24.39	−23.75	−24.18	−27.67	−27.93	−27.89	−28.46	−23.69	−24.34
S.D.	0.16	0.17	0.27	0.34	0.32	0.34	0.25	0.35	0.31	0.59	0.26	0.28
CV (%)	0.7	0.7	1.1	1.4	1.4	1.4	0.9	1.2	1.1	2.1	1.1	1.2
Minimum (‰)	−24.59	−24.35	−24.83	−25.00	−24.27	−24.70	−28.13	−28.66	−28.52	−29.30	−24.30	−25.07
Maximum (‰)	−23.84	−23.75	−23.80	−23.81	−23.02	−23.73	−27.46	−27.43	−27.12	−27.13	−23.17	−23.74

ranging from 20 to 200 ng. The purpose of the quality controls is to verify the reproducibility of the extraction procedure and GC/C/IRMS measurements. The within-assay precision was determined by extracting five aliquots of QC-Pos and QC-Neg and injecting each once within the same day. For the determination of the between-assay precision, 16 aliquots of QC-Pos and QC-Neg were extracted by four different technicians over a period of 12 months, and injected once each. The within-assay precision and the between-assay precision for all steroids of interest in QC-Pos and QC-Neg are listed in Tables 1 and 2, respectively. These results indicate that the S.D.s for each compound measured with QC-neg and QC-pos are slightly higher in the case of the between-assay precision (range: 0.16–0.59‰) compared to the within-assay precision (range: 0.16–0.29‰). Although this method allows for the δ^{13}C-value determination of six steroids, the S.D.s and CVs are comparable to existing IRMS methods used for two or three steroids [20,21]. For QC-Pos in the within-assay precision, it is worth noting that the isotopic mean values of androstenol and 5βP are comparable and differ by at least 3.5‰ with the values of the T metabolites (Table 1). These findings tend to indicate that the isotopic values of androstenol are likely not affected by testosterone ingestion.

3.2. Concentration and δ^{13}C-values of androstenol and 5βP in a reference population

To investigate the potential use of androstenol as an endogenous reference compound, the concentration of both androstenol and 5βP were determined and compared in fraction F2, without correction for the recovery yields. The limits of detection (LOD) and quantification (LOQ) of the method used for quantification of androstenol and 5βP in fraction F2 were of 0.3 and 1.2 ng/μl, respectively. Fig. 3 shows the concentration of androstenol and 5βP extracted from the urines of a healthy population composed of 10 females and 10 males of Caucasian origin living in Switzerland. The concentration ratio androstenol/5βP vary from 1.3 to 9.7 with a mean of 4.5 (S.D. = 2.8, CV = 61%) for the male population with an age range of 22–34 years, whereas the ratio values range from to 0.1 to 1.4 with a mean of 0.6 (S.D. = 0.4, CV = 59%) for the female population with an age range of 21–35 years. Although more data are needed to have a more descriptive statistic, these findings significantly show that the concentration of androstenol is higher than 5βP in the male population. Except for one subject, the ratio is lower than 1.0 in the female population. For two subjects, the ratio androstenol/5βP is of 0.1, with a much higher concentration of 5βP compared to the other subjects. Indeed, it is known that a strong increase of the 5βP-excretion can be observed in the second part of the female menstrual cycle due to the progesterone production of the corpus luteum with consecutive metabolism of progesterone to 5βP [22].

As mentioned previously, 5β-pregnane-3α,20α-diol is used as an endogenous reference compound in steroid IRMS measurements. To validate the use of androstenol as an endogenous reference, the δ^{13}C-values of androstenol and 5βP were therefore compared for the urine of the reference population. The δ^{13}C-values of androstenol and 5δP for the male and female populations are depicted in Fig. 4. The differences of the intra-individual δ^{13}C-values for androstenol and 5δP are less than 0.9‰ for both populations. In the male population, the mean δ^{13}C-values are of −23.29 and −23.18‰ for androstenol and 5βP, respectively, whereas −23.69 and −23.71‰ are obtained for the female population. These findings show that the mean δ^{13}C-values are similar for both population and are comparable to isotopic values of endogenous substances of healthy subjects living in Europe [15].

USADA 0794

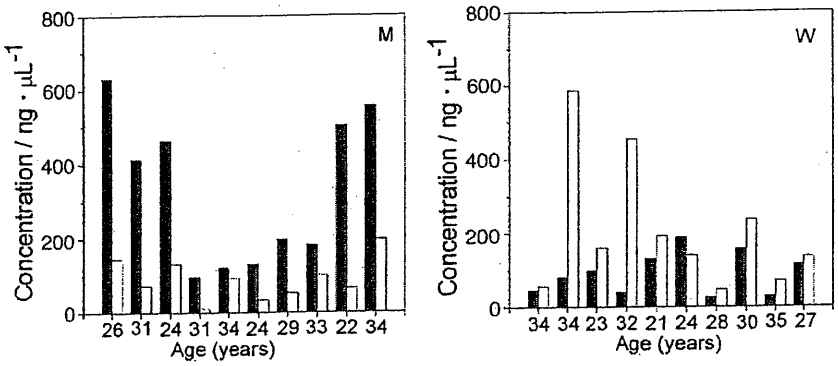


Fig. 3. Concentration in fraction F2 of androstenol (dark column) and 5βP (white column) extracted from the urines of a healthy population composed of 10 females (W) and 10 males (M) of Caucasian origin. The dark and white columns depict the androstenol and 5βP concentrations, respectively.

The S.D.s for androstenol and 5βP are comparable in the male population ($S.D._{androstenol}$ = 0.45‰, $S.D._{5\beta P}$ = 0.46‰) and slightly lower than the S.D.s of both steroids in the female population ($S.D._{androstenol}$ = 0.83‰, $S.D._{5\beta P}$ = 0.79‰).

Since the 5βP δ^{13}C-values are not affected by testosterone or DHEA administration [7,10], this compound is frequently used as an endogenous reference. The comparison of the concentration and isotopic values between androstenol and 5βP in a control group has shown no significant isotopic discrimination for androstenol and sufficient amounts for IRMS analysis. Therefore, these findings together with evidences of androstenol biosynthesis demonstrate the potential use of this compound as an endogenous reference for detecting doping with testosterone or DHEA.

3.3. δ^{13}C-values following oral DHEA and pregnenolone administration

The method was applied in the frame of an excretion study following oral ingestion of 50 mg DHEA initially and oral ingestion of 50 mg pregnenolone 48 h later by a Caucasian healthy male volunteer. The time course of isotopic ratio values in urine for the steroids of interest is shown in Fig. 5. Baseline δ^{13}C-values were obtained from two urines collected 26 and 2 h before DHEA administration.

As reported in a previous study [10], the δ^{13}C-value of 5βP was not affected by administration of DHEA. In the seven urines measured during the DHEA excretion study (t = 0–48 h), we determined a mean δ^{13}C-value of −23.71‰ with S.D. = 0.23‰, range: −23.38 to −24.05‰ for 5βP. The S.D. value is similar to what obtained for the between-assay of QC-neg and QC-pos and shows that the δ^{13}C-values for 5βP remain constant during the time course of the DHEA excretion. During this time course, the δ^{13}C-values of androstenol range from −23.54 to −24.10‰ with a mean δ^{13}C-value = −23.89‰ and a S.D. = 0.20‰. These findings show similar results for androstenol and 5βP, and therefore no significant variation of the isotopic values of both steroids upon DHEA ingestion. However, significant changes of the isotopic values of the T metabolites were observed after ingestion of DHEA. Fig. 5 illustrates these changes with comparable minimum δ^{13}C-value of −29.98, −30.41, −29.73 and −30.19‰ for androsterone, etiocholanolone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol, respectively. Thus, a difference higher than 6‰ is detected for the T metabolites with respect to the mean δ^{13}C-value of androstenol and 5βP after ingestion of DHEA. For the spot urine at t = 36 h, it may be still observed a difference of 0.4, 1.6, 3.8, 4.4‰ for androsterone, etiocholanolone, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, respectively, com-

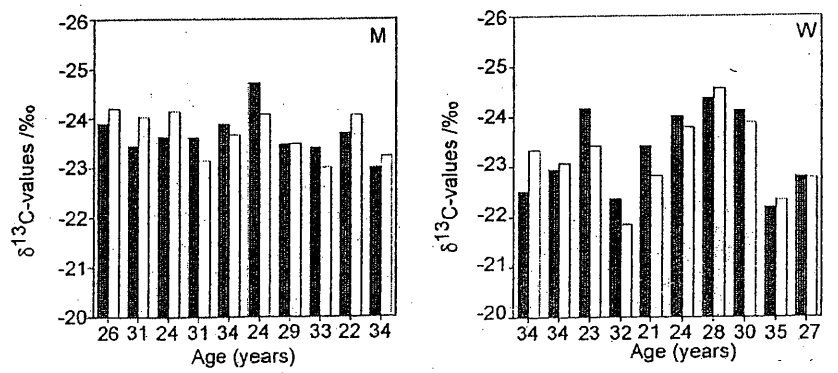


Fig. 4. The δ^{13}C values of androstenol (dark column) and 5βP (white column) extracted from the urines of a healthy population composed of 10 females (W) and 10 males (M) of Caucasian origin. The dark and white columns depict the androstenol and 5βP δ^{13}C-values, respectively.

USADA 0795

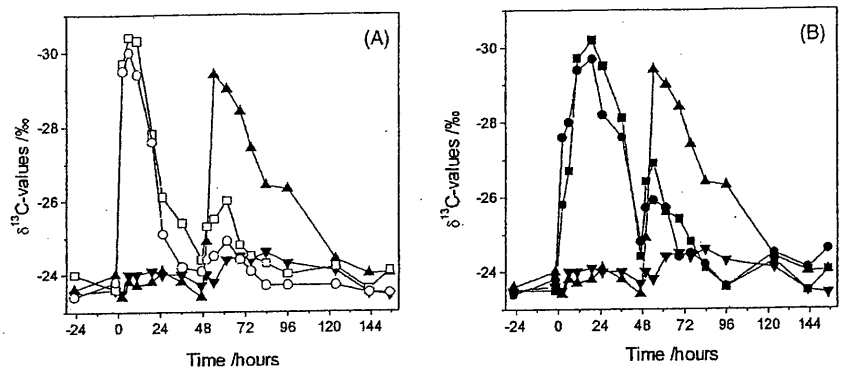


Fig. 5. Excretion study of DHEA (50 mg) and pregnenolone (50 mg), ingested initially ($t = 0$) and 48 h later ($t = 48$ h), respectively. Panel A represent the time course of the urinary δ^{13}C-values of andro (□), etio (○) together with 5βP (▲) and androstenol (▼), whereas panel B shows the time course of the urinary δ^{13}C-values of 5αA (■) and 5βA (●) together with 5βP (▲) and androstenol (▼).

pared to the δ^{13}C-value of the endogenous reference 5βP.

After ingestion of 50 mg of pregnenolone, 10 spot urines were collected over a period of 105 h. The δ^{13}C-values of 5βP were significantly affected with a difference of 5.69‰ compared to the mean δ^{13}C-value of 5βP of the urines collected during 48 h after DHEA ingestion. Similar results with δ^{13}C-values variations of 5βP higher than 4‰ were obtained on four subjects upon oral administration of 50 mg of pregnenolone [18]. As depicted in Fig. 5, the δ^{13}C-values returned to the baseline 75 h after pregnenolone ingestion. Such a difference in the carbon isotope ratio value reveals the synthetic origin of the steroid and it tends to demonstrate that 5βP is not longer applicable as endogenous reference compound. As represented in Fig. 1, pregnenolone may be also transformed to androstenol and to testosterone through the pathways leading to the formation of 16-ene steroids and sex steroids, respectively. The results show that the difference in the isotopic values of androstenol were lower than 1‰ compared to the mean value obtained after DHEA ingestion (from $t = 0$ to 48 h), while for androsterone, etiocholanolone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol it may be observed minimum δ^{13}C-values of −24.95, −26.00, −25.87 and −26.86‰, respectively. Thus, the isotopic values of the T metabolites have changes lower than 3‰ after oral administration of 50 mg pregnenolone.

After pregnenolone ingestion, the largest variation of the δ^{13}C-values was obtained with 5βP. Although pregnenolone is a precursor for all investigated steroids, similar variation of the δ^{13}C-values for all of them were not observed. In a previous study, it has been reported no influence of the isotopic values of androsterone, etiocholanolone and 5β-androstane-3α,17β-diol upon administration of pregnenolone [18]. Hence, these findings tend to demonstrate a major conversion of pregnenolone to 5βP compared to the T metabolites (sex steroids pathway) or androstenol (16-ene steroids pathway). Although other excretion studies on different subjects with multiple ingestions at variable dose schedules would be needed to test this effect, our results show that androstenol may serve as endogenous reference compound after ingestion of pregnenolone.

In conclusion, we have presented an IRMS method for the determination of the δ^{13}C-values of four T metabolites together with 5βP and androstenol. This method gives consistent and reproducible δ^{13}C-values for the analyzed steroids. Our findings show that 5βP and androstenol may be used as endogenous reference compounds when DHEA or testosterone has been administered. However, administration of pregnenolone will significantly affect the isotopic values of 5βP, with much smaller variations of the androstenol δ^{13}C-values. Therefore, it may be anticipated that the differences between the δ^{13}C-values of androstenol and 5βP could be an indication of pregnenolone administration.

Acknowledgements

This study was supported by a grant from the Swiss Olympic Association. We thank A. Maître for fruitful discussions and S. Cruchon for technical assistance.

References

[1] D.B. Gower, B.A. Ruparelia, J. Endocrinol. 137 (1993) 167, and references therein.
[2] J.J. Weusten, G. Legemaat, M.P. van der Wouw, A.G. Smals, P.W. Kloppenborg, T. Benraad, J. Steroid Biochem. 32 (1989) 689.
[3] P. Soucy, L. Lacoste, V. Luu-The, Eur. J. Biochem. 270 (2003) 1349.
[4] I. Dufort, P. Soucy, L. Lacoste, V. Luu-The, J. Steroid Biochem. 77 (2001) 223.
[5] M. Morofushi, K. Shinohara, T. Funabashi, F. Kimura, Chem. Senses 25 (2000) 407.
[6] B.F. Forman, I. Tzameli, H.-S. Choi, J. Chen, D. Simha, W. Seol, R.M. Evans, D.D. Moore, Nature 395 (1998) 612.
[7] R. Aguilera, D.H. Catlin, M. Becchi, A. Phillips, C. Wang, R.S. Swerdloff, H.G. Pope, C.K. Hatton, J. Chromatogr. 72 (1999) 95.
[8] C.H. Shackleton, A. Phillips, T. Chang, Y. Li, Steroids 62 (1997) 379.
[9] S. Hornig, H. Geyer, M. Machnik, W. Schänzer, A. Hilkert, J. Oebelmann, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-

USADA 0796

Engelke (Eds.), Recent Advances in Doping Analysis, Sport und Buch Strauss, Köln, 1996, p. 275.
[10] M. Ueki, M. Okano, Rapid Commun. Spectrom. 13 (1999) 2237.
[11] R. Aguilera, C.K. Hatton, D.H. Catlin, Clin. Chem. 48 (2002) 629.
[12] C.H. Shackleton, E. Roitman, A. Phillips, T. Chang, Steroids 62 (1997) 665.
[13] S. Horning, H. Geyer, U. Flenker, W. Schänzer, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Sport und Buch Strauss, Köln, 1997, p. 135.
[14] J.-C. Mathurin, V. Herrou, E. Bourgogne, L. Pascaud, J. de Ceaurriz, J. Chromatogr. B 759 (2001) 267.
[15] E. Bourgogne, V. Herrou, J.-C. Mathurin, M. Becchi, J. de Ceaurriz, Rapid Commun. Spectrom. 14 (2000) 2342.
[16] U. Flenker, W. Schänzer, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Sport und Buch Strauss, Köln, 2003, p. 179.
[17] C. Ayotte, D. Gouldreault, A. Lajeunesse, M. Cléroux, Y. Richard, A. Charlebois, J.-P. Couture, A. Fakirian, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Sport und Buch Strauss, Köln, 2001, p. 133.
[18] M. Ferry, J.-C. Mathurin, M. Becchi, J. de Ceaurriz, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Sport und Buch Strauss, Köln, 2000, p. 209.
[19] D.M. Jones, J.F. Carter, G. Eglinton, E.J. Jumeau, C.S. Fenwick, Biol. Mass Spectrosc. 20 (1991) 641.
[20] R. Aguilera, T.E. Chapman, B. Starcevic, C.K. Hatton, D.H. Catlin, Clin. Chem. 47 (2001) 292.
[21] R. Aguilera, T.E. Chapman, D.H. Catlin, Rapid Commun. Spectrom. 14 (2000) 2294.
[22] U. Mareck-Engelke, H. Geyer, W. Schänzer, in: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Sport und Buch Strauss, Köln, 1997, p. 51.

USADA 0797

Urinary Analysis of Four Testosterone Metabolites and Pregnanediol by Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry after Oral Administrations of Testosterone

Arnaud Maître, Christophe Saudan*, Patrice Mangin, and **Martial Saugy**

Laboratoire Suisse d'Analyse du Dopage, Institut Universitaire de Médecine Légale, Rue du Bugnon 21, 1005 Lausanne, Switzerland

Abstract

The most frequently used method to demonstrate testosterone abuse is the determination of the testosterone and epitestosterone concentration ratio (T/E ratio) in urine. Nevertheless, it is known that factors other than testosterone administration may increase the T/E ratio. In the last years, the determination of the carbon isotope ratio has proven to be the most promising method to help discriminate between naturally elevated T/E ratios and those reflecting T use. In this paper, an excretion study following oral administration of 40 mg testosterone undecanoate initially and 13 h later is presented. Four testosterone metabolites (androsterone, etiocholanolone, 5α-androstanediol, and 5β-androstanediol) together with an endogenous reference (5β-pregnanediol) were extracted from the urines and the $\delta^{13}C/^{12}C$ ratio of each compound was analyzed by gas chromatography–combustion–isotope ratio mass spectrometry. The results show similar maximum $\delta^{13}C$-value variations (parts per thousand difference of $\delta^{13}C/^{12}C$ ratio from the isotope ratio standard) for the T metabolites and concomitant changes of the T/E ratios after administration of the first and the second dose of T. Whereas the T/E ratios as well as the androsterone, etiocholanolone and 5α-androstanediol $\delta^{13}C$-values returned to the baseline 15 h after the second T administration, a decrease of the 5β-androstanediol δ-values could be detected for over 40 h. This suggests that measurements of 5β-androstanediol δ-values allow the detection of a testosterone ingestion over a longer post-administration period than other T metabolites $\delta^{13}C$-values or than the usual T/E ratio approach.

Introduction

The detection of doping with testosterone is mainly based on the concentration measurement of the testosterone (T) to epitestosterone (E) ratio (T/E ratio). If this T/E ratio is greater than 6 in the urine of a competitor, it is mandatory that additional investigations should be conducted. Isotope ratio mass spectrometry (IRMS) may be used in this context as an additional analysis to help in deciding whether an elevated T/E ratio can be explained by a misuse of testosterone (1–4) or testosterone precursors (4,5). Indeed, it is known that other factors than testosterone administration may lead to T/E ratio exceeding 6 (6,7).

IRMS allows measurements of slight differences in the carbon isotope ratio ($^{13}C/^{12}C$) of the exogenous and endogenous testosterone. Synthetic testosterone is produced from precursors derived from plants with low ^{13}C content, whereas the ^{13}C and ^{12}C content in the natural endogenous form depends on the isotopic carbon composition of the food diet and is influenced by additional effects of human biological processing (8). Such differentiation between synthetic and endogenous origins has also been reported for other hormones such as epitestosterone (9), nandrolone (10), and corticosteroids (11).

Very few time profile IRMS studies with the chronological determination of T/E ratio after oral testosterone administration are reported in the literature (2,3,12). Chronological measurement of the carbon isotopic ratio of T metabolites was able to detect T administration in cases in which the T/E ratio remained below 6 (2). It must be noted that in this latter study the five volunteers were of Chinese ethnicity, and therefore, the cutoff limit of a T/E ratio of 6 that is suited for the caucasian race is questionable when used on a Chinese subject population. It is also worth mentioning that the excretion studies on two caucasian volunteers after one oral ingestion of T undecanoate (3). These excretion studies report the detection of exogenous T by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) after the T/E ratio had returned to below 6. The determination of exogenous T with GC–C–IRMS was possible for more than twice as long as with application of T/E ratio limits.

* Author to whom correspondence should be addressed: Laboratoire Suisse d'Analyse du Dopage, Institut Universitaire de Médecine Légale, Rue du Bugnon 21, 1005 Lausanne, Switzerland. E-mail: csaudan@hospvd.ch.

USADA 0798

This paper describes the time courses of isotopic ratio values in urine of androsterone (Andro), etiocholanolone (Etio), 5α-androstanediol (5αA), 5β-androstanediol (5βA), and the endogenous reference 5β-pregnanediol (5βP) in the frame of an excretion study following oral ingestion of testosterone initially and 13 h later by a healthy, male, caucasian volunteer (Figure1). The profile of the T/E ratio is also compared with the results obtained by IRMS. These excretion studies are fundamental in understanding how the time profile of IRMS values is affected after multiple oral ingestions of testosterone. To our knowledge, there is no report of chronological application of the isotope ratio technology and the T/E ratio to monitor an excretion study with multiple oral testosterone ingestions.

Experimental

Chemicals

All solvents and reagents of analytical grade purity were purchased from Fluka (Buchs, Switzerland). 5α-Androstan-3β-ol acetate, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 5β-pregnane-3α,20α-diol, etiocholanolone, and androsterone with a purity of at least 99.5% were obtained either from Steraloids Inc. (Newport, RI) or Sigma Chemical Co. (St. Louis, MO). Bakerbond spe™ 500-mg or 1000-mg octadecyl C_{18} disposable extraction columns were obtained from J.T. Baker (Phillipsburg NJ). β-Glucuronidase from *Escherichia coli* in a 50% glycerol solution (pH 6.5, 140 U/mL at 37°C) was supplied by Roche Diagnostics GmbH (Mannheim, Germany). The reference carbon dioxide gas was purchased from Carbagaz (Domdidier, Switzerland). The mixture of three alkanes, C_{15} (*n*-pentadecane), C_{20} (*n*-eicosane), and C_{25} (*n*-pentacosane), was supplied by Chiron AS (Trondheim, Norway).

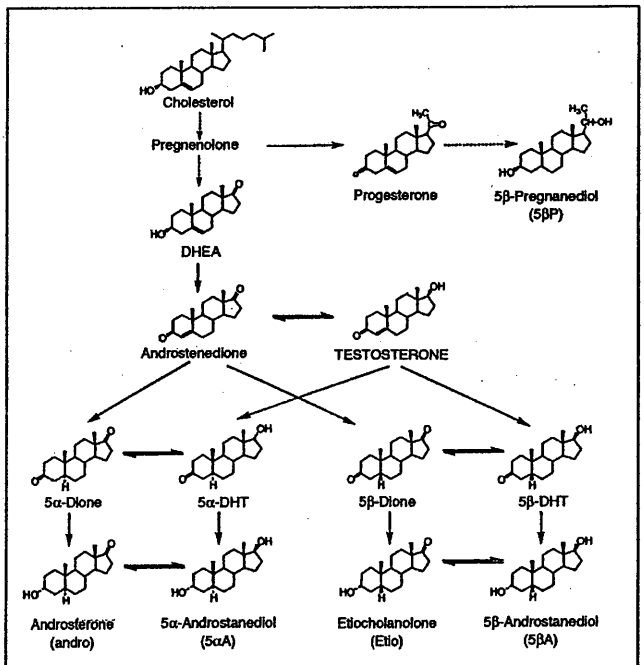


Figure 1. Metabolic pathways of testosterone.

Dosage and collection of urine samples

A 40-mg testosterone undecanoate capsule (Panteston®, Organon SA, France) were self-administered orally initially and 13 h later by a healthy, male, caucasian volunteer. Baseline urine samples were obtained before initial administration, and 39 subsequent spot urine samples were collected over a period of 100 h after the first capsule administration. Reference urine samples were collected from 40 caucasian male subjects. In this excretion study, the spot urine with the T/E ratio of 36 at t = 2.3 h was selected to be the positive quality control urine (QC-pos). The negative quality control urine (QC-neg) was obtained from a healthy subject. Each urine was aliquoted into 15-mL cryogenic tubes and stored without additives at –20°C until analysis.

Sample preparation

The urine sample (10 mL) was centrifuged in glass tubes at 2500 rpm for 5 min and was applied onto a C_{18} column (500 mg). The conjugated steroids were eluted with methanol (8 mL) and the eluate evaporated to dryness. To hydrolyze the conjugated steroids, the residue was dissolved in 1 mL of 0.2M phosphate buffer (pH 7.0) and 50 µL of β-glucuronidase was added as supplied. After incubation at 50°C during 1 h (or at 37°C overnight) in a thermostated water bath, the deconjugated steroids were extracted on C_{18} column (500 mg) by washing with 6 mL acetonitrile/H_2O (20:80, v/v) and subsequently by 6 mL acetonitrile/H_2O (35:65, v/v) and finally eluted with 12 mL acetonitrile. The eluate was evaporated to dryness under a nitrogen stream (Turbo Vap LV evaporator, Zymark, Hopkinton, MA), and then the residue was dried over phosphorus pentoxide for 20 min. Acetylation of the extract was carried out in 50 µL of pyridine and 50 µL of acetic anhydride at 60°C for 1 h incubation. The reaction medium was evaporated to dryness and subsequently the residue was redissolved in 3 mL of acetonitrile/H_2O (50:50, v/v). The solution was applied onto a C_{18} column (1000 mg). The fraction F1 containing androsterone and etiocholanolone was obtained by elution with 15 mL acetonitrile/H_2O (75:25, v/v) after washing with 12 mL of acetonitrile/H_2O (50:50, v/v). Finally, the fraction F2 with the androstandiols and the pregnanediol was obtained by eluting with 12 mL of acetonitrile. The derivatized steroids of fractions F1 and F2 were spiked with 40 µL and 10 µL of an internal standard (IS, 5α-androstan-3β-ol acetate, 0.20 mg/mL in methanol), respectively. Then both fractions F1 and F2 were evaporated to dryness and finally dissolved in 500 µL and 40 µL of cyclohexane, respectively.

GC–C–IRMS analysis

The carbon isotope measurements were performed on a Delta^{Plus} IRMS system (Thermo Finnigan MAT, Bremen, Germany) coupled to an Agilent 6890A GC (HP Analytical Division, Waldbronn, Germany) via a Finnigan GC Combustion III interface (Thermo Finnigan MAT, Bremen, Germany) and a CTC Analytics CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland). Chromatographic separations were achieved on an HP cross-linked 50% phenylmethylsiloxane fused silica capillary column (J&W Scientific, 30 m × 0.25-mm i.d., 0.15-µm film thickness). The injector temperature was set to 280°C.

The combustion and reduction oven temperatures were set to 940°C and 600°C, respectively. Reference carbon dioxide gas pulses (20-s durations) were introduced at four different times during the course of the chromatographic separation. For the calibration of the reference gas with the alkane mixture of known $\delta^{13}C$-values, the oven temperature was increased from 80°C (1 min) to 270°C (7.0 min) at 15°C/min. For the analysis of the androsterone and etiocholanolone acetates (fraction F1), the oven temperature was increased from 80°C (1 min) to 270°C (8.3 min) at 15°C/min, then to 300°C at 35°C/min, and maintained at the final temperature for 3 min. For the analysis of the fraction F2 containing the androstandiols and the pregnanediol acetates, the oven temperature was increased from 80°C (1 min) to 270°C (11.5 min) at 15°C/min, then to 300°C at 35°C/min, and maintained at the final temperature for 3 min. The volume of injection was 1–2 µL, depending on the estimated concentrations of the compounds of interest as evaluated in the frame of the T/E ratio determination. The extracts were injected twice in the splitless mode, and the replicate measurements were averaged.

The symbol δ is the standard notation for expressing carbon isotope ratios. It is defined as parts per thousand deviation of isotopic compositions from that of Pee Dee Belemnite (PDB), and is calculated according to:

$$\delta^{13}C/‰ = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}}$$

Negative shifts of the $\delta^{13}C$-value due to the formation of an acetate were corrected as follows:

$$D_{OH} = D_{OAC} + 2m\,\frac{(D_{OAC} - D_{AC})}{n}$$

Where D_{OH} is $\delta^{13}C$-value for the underivatized steroids, D_{OAC} is the $\delta^{13}C$-value for the acetylated steroids, D_{AC} is the $\delta^{13}C$-value for the acetylating reagent, n is the number of carbon atom in a molecule, and m is the number of hydroxyl groups to be acetylated.

All subsequent $\delta^{13}C$-values have been corrected for this negative shift. The D_{AC} value of our acetylating reagent was determined to be –48.8‰ according to a published procedure (13).

Determination of steroid concentration and T/E ratio

Urinary testosterone and epitestosterone were quantified in all the samples and the concentration of the steroids of interest were quantified according to the method of Nolteemsting et al (14).

Results and Discussion

Isotope measurements, accuracy, and reproducibility of the method

The reference carbon dioxide gas, which is pulsed several times during the course of the chromatographic separation, is calibrated by a mixture of three alkanes, C_{15} (*n*-pentadecane), C_{20} (*n*-eicosane), and C_{25} (*n*-pentacosane), from Chiron AS with $\delta^{13}C$-values of –30.22, –33.06, and –28.21‰, respectively. The internal standard (5α-androstan-3βol acetate) was measured together with this alkane mixture and the $\delta^{13}C$ value was –31.9‰ (n = 50, SD = 0.2‰).

The extraction method allows the separation of androsterone (Andro) and etiocholanolone (Etio) acetates from the 5α-androstanediol (5αA), 5β-androstanediol (5βA), and pregnanediol (5βP) acetates on the C_{18} column by eluting with different ratio mixture of acetonitrile/H_2O (15). Before the GC–C–IRMS analysis, an internal standard (5α-androstan-3β-ol acetate) with a known $\delta^{13}C$-value is spiked in both fractions. Figure 2 shows an example of GC–C–IRMS chromatograms of *m/z* 44 for fractions F1 and F2. The chromatograms show symmetrical peaks for the compounds of interest, no tailing, and no evidence of co-eluting compounds.

Each batch includes positive (QC-Pos) and negative (QC-Neg) quality controls and the measurements are performed for all metabolites in the linear range of the IRMS. The purpose of the quality controls is to verify the reproducibility of the extraction procedure and the GC–C–IRMS measurements. The two incubation procedures given in the experimental part have been verified with the positive and negative quality controls to

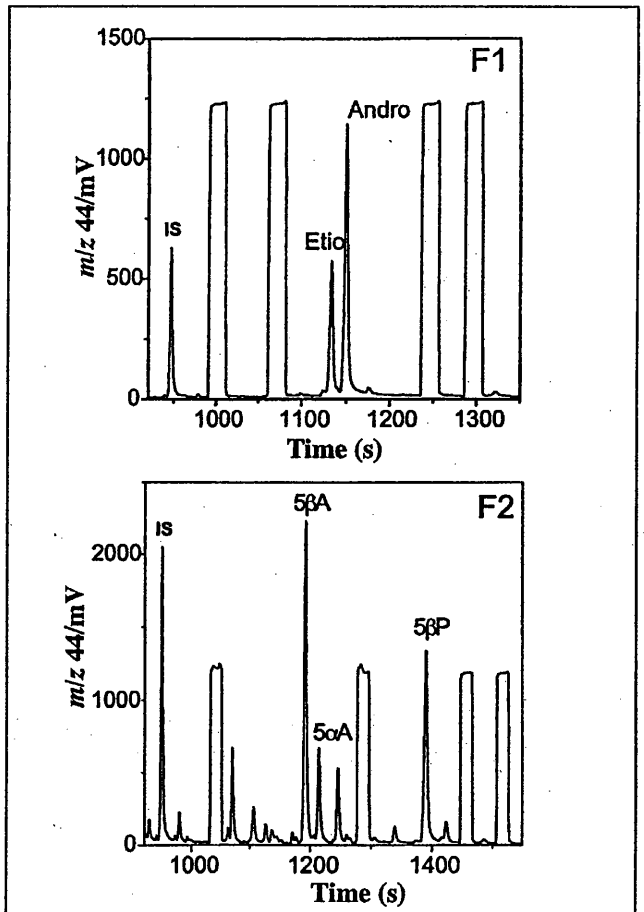


Figure 2. Typical GC–C–IRMS *m/z* 44 mass chromatograms of fractions F1 (androsterone and etiocholanolone acetates) and F2 (5α-androstanediol, 5β-androstanediol, and 5β-pregnanediol acetates) spiked with an internal standard (IS, 5α-androstan-3β-ol acetate). The square-topped peaks represent pulses of CO_2 reference gas.

give comparable results. The within-assay precision was determined by extracting six aliquots of QC-Pos and QC-Neg and injecting each once within the same day. For the determination of the between-assay precision, 20 aliquots of QC-Pos and QC-Neg were extracted by 4 different technicians over a period of 18 months, and injected once each. For the within-assay precision the overall means and SD for etiocholanolone, androsterone, 5β-androstanediol, 5α-androstanediol, and 5β-pregnanediol in the QC-neg were –24.6 (± 0.1), –24.2 (± 0.2), –23.9 (± 0.1), –23.6 (± 0.3), and –23.2 (± 0.2) ‰, respectively, whereas in the QC-pos, –27.9 (± 0.3), –27.7 (± 0.2), –28.2 (± 0.2), –28.0 (± 0.2), and –23.2 (± 0.2) ‰ were obtained. For the between-assay precision, the overall means and SD for etiocholanolone, androsterone, 5β-androstanediol, 5α-androstanediol, and 5β-pregnanediol in the QC-neg were –24.4 (± 0.4), –24.0 (± 0.4), –24.3 (± 0.3), –24.3 (± 0.4), and –23.7 (± 0.4) ‰, respectively. For the QC-pos, means and SDs of –27.8 (± 0.4), –27.9 (± 0.4), –27.8 (± 0.4), –28.4 (± 0.5), and –23.2 (± 0.3) ‰ were obtained. These results indicate that the SDs for each compound measured with QC-neg and QC-pos are slightly higher in the case of the between-assay precision compared to the within-assay precision. Although this method allows for the δ^{13}C-value determination of five steroids, the SDs are comparable to existing IRMS methods used for two T metabolites (12,16).

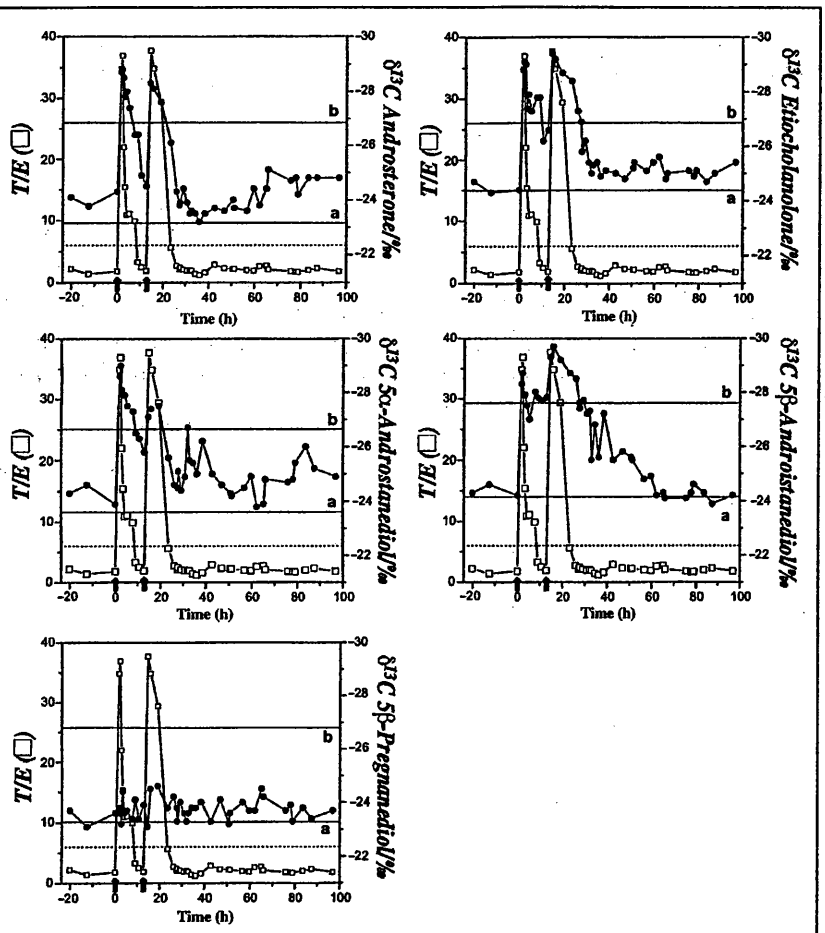


Figure 3. δ_{13}C-Values of androsterone, etiocholanolone, 5α-androstanediol, 5β-androstanediol, and 5β-pregnanediol together with the T/E ratio throughout the study period. Testosterone was administered at $t = 0$ and 13 h later ($t = 13$). The dotted line displays T/E ratio = 6. The horizontal lines a and b represent the δ^{13}C-values for the mean and the mean – 3SD, respectively.

Table I. Absolute δ^{13}C-Values of Androsterone, Etiocholanolone, 5αA, 5βA, and 5βP of a Healthy, Caucasian, Male Population*

	Mean		SD		Range		Median
δ^{13}C-value /‰	This study	ref 5	This study	Ref 5	This study	Ref 5	this study
Androsterone	–23.2	–	1.23	–	–21.2 to –25.3	–	–23.2
Etiocholanolone	–24.4	–	0.83	–	–22.1 to –25.8	–	–24.6
5αA	–23.6	–17.5	1.00	3.53	–21.8 to –25.9	–10.5 to –24.6	–23.8
5αA	–24.2	–20.0	1.15	2.75	–22.0 to –26.3	–15.2 to –24.6	–24.3
5αP	–23.3	–21.0	1.17	1.65	–20.7 to –25.6	–17.2 to –23.8	–23.2

* ($n = 40$). Data obtained by Ueki and Okano (5) for an athlete population of males and females of various ethnicities ($n > 350$).

δ^{13}C Values following oral testosterone administration

The GC–C–IRMS and the T/E ratio results from the excretion study following oral administration of 40 mg testosterone undecanoate initially and 13 h later is shown in Figure 3. Figure 3 also shows the mean and the mean-3SD δ^{13}C-values for androsterone, etiocholanolone, 5αA, 5βA, and 5βP obtained after analysis of a reference population of healthy caucasian males ($n = 40$). Table I lists also the mean δ^{13}C-values, the median δ^{13}C-values, the SDs, the δ^{13}C range values of androsterone, etiocholanolone, 5αA, 5βA, and 5βP, together with the results obtained by Ueki and Okano (5) for 5αA, 5βA, and 5βP from a population of 450 athletes who had participated to the XVII Olympic Winter Games in Nagano (Japan). This population with a majority of Caucasian subjects may be compared to our data because a correction factor was used to convert all measured δ^{13}C-values of the acetylated compounds to give values for underivatized steroids. A correction factor was not applied for the population studies obtained by Shakelton et al. (2) and Aguilera et al. (16). As listed in Table I, only 5αA, 5βA, and 5βP may be compared with the data of Ueki and Okano (5). There are striking differences between the ranges found in the two laboratories with mean differences of 6.1, 4.2, and 2.3‰ for 5αA, 5βA, and 5βP, respectively. Moreover, our SDs are much lower to those obtained by Ueki and Okano (5). These results may be explained by a difference of the analytical method or from the diet heterogeneity of the population. Indeed, the diet of subjects from Europe is different from that of America and Japan and before the XVII

Olympic Winter Games in Nagano (Japan), the athletes did not eat the local diet for a sufficient time to have a measurable change of the steroids $\delta^{13}C$-values in urine. Differences to ethnicity may not explain the ranges of the $\delta^{13}C$-values for 5αA, 5βA, and 5βP in a population (2,16).

As reported in previous studies (2,8), the $\delta^{13}C$-value for 5bP should not be affected by the administration of testosterone since it has to be produced from cholesterol via several intermediates (Figure 1). A change of the $\delta^{13}C$-value for 5βP and for T metabolites could be expected if the individual modify drastically the diet during the study period. In the 41 urines collected during the excretion study, we determined a mean $\delta^{13}C$-value of –23.7‰ with SD = ± 0.4‰, range: –22.6 to –24.6‰ and median = –23.7‰ for 5βP. The SD value is similar to what obtained with the between-assay of QC-neg and QC-pos and shows that the $\delta^{13}C$-values for 5βP remain constant during the time course of the excretion. These findings reveal no dietarymodification during the excretion study and are in agreement with previous investigations (2,8).

Interestingly, T/E ratio reach a maximum of 36 after both administrations and this value is higher than what has been reported (3) after an oral dose of 40 mg testosterone undecanoate for two caucasian individuals (T/E ratio = 22). These differences may be rationalized as an interindividual variability in metabolism (17), such as genetic differences in the cytochrome P450 system (7) and preferential biotransformation of the steroids. Differences in volume distribution and body weights may also explain these results.

It is noteworthy that the T/E ratio increased rapidly following testosterone administration but returned below 6 after 10 and 15 h for the first and second T administrations, respectively (Figure 3). Therefore only the urines collected during this period would be considered as suspect and subject to further procedures (6). Carbon isotope ratio measurements are also used for that purpose in detecting testosterone abuse. Similarly to the T/E ratio, the $\delta^{13}C$-values of the four T metabolites decrease rapidly after T administration with a difference of about 5‰ with respect to the endogenous reference 5βP. These findings together with the fact that the values are out of the range as defined by the population mean – 3SD for all T metabolites would be strong indicators of testosterone abuse. Indeed, a difference superior to 4‰ with respect to the endogenous reference 5βP is indicative of an exogenous T ingestion (8,16). This difference of 4‰ is slightly higher to 3SD for all T metabolites of the reference population (Table I).

The results show similar maximum $\delta^{13}C$-value variations for the T metabolites and concomitant changes of the T/E ratios after administration of the first and the second dose of T. Whereas androsterone, etiocholanolone, and 5α-androstanediol δ-values returned to the baseline 15 h after the second T administration, a decline in the 5α-androstanediol δ-values could be detected over 40 h. This finding may be related to different metabolic pathways with different kinetics from testosterone to T metabolites. For instance, it is known that urinary 5α-androstanediol arise from both hepatic and peripheral metabolism whereas urinary 5β-androstanediol is considered to arise only from hepatic metabolism (16). One should also consider the wash out period between the administration of the first and the second dose. About 10.3 h after the second T dose, it may be observed on the T/E ratio profile that there is likely no residual effect on this parameter from the first dose, whereas it may not be excluded from the 5β-androstanediol and ethiocholanolone $\delta^{13}C$-values. Other excretion studies on different subjects with multiple T ingestions are variable dose schedules would be needed to test this effect.

It may be also emphasized that after ingestion of the second dose of T, there is a spot urine with a T/E ratio below 6 (T/E = 5.6 at t = 23.3 h) that shows for androsterone, etiocholanolone, 5α-androstanediol, and 5β-androstanediol a difference of 2.3, 4.6, 1.8, and 4.9‰ respectively, with the $\delta^{13}C$-value of the endogenous reference 5βP. For the subsequent spot urine at t = 26 h, a T/E ratio of 2.7 was obtained with a $\delta^{13}C$-value superior to 4‰ for 5β-androstanediol. From these results and other studies (3,12), it may be asked in routine procedure: 1. If the T/E ratio at 6 is still a valid cutoff and 2. What are the IRMS criteria to declarean exogenous T ingestion, that is, does all the measured T metabolite $\delta^{13}C$-values or does only one have to be superior to 4‰. Further excretion studies with multiple T ingestions should help in the future in deciding which criteria should be unilaterally adopted.

In conclusion, our results suggest that measurements of 5β-androstanediol δ-values allow the detection of a testosterone ingestion over a longer postadministration period than other T metabolites $\delta^{13}C$-values or than the usual T/E ratio approach. A difference of 4‰ with respect to the endogenous reference 5βP should be used as indicative of an exogenous T ingestion. As discussed previously, the use of a reference population to report positive samples is questionable.

Acknowledgment

This study was supported by a grant from the Swiss Olympic Association. We thank C. Schweizer for fruitful discussions and S. Cruchon for technical assistance.

References

1. R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic, and H.G. Pope. Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom.* **31:** 169–176 (1996).
2. C.H. Shackleton, A. Phillips, T. Chang, and Y. Li. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstandiols. *Steroids* **62:** 379–387 (1997).
3. S. Hornig, H. Geyer, M. Machnik, W. Schänzer, A. Hilkert, and J. Oebelmann. Detection of exogenous testosterone by $^{13}C/^{12}C$ analysis. In *Recent advances in Doping Analysis (4)*, M. Donike, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, Germany, 1996, pp 275–284.
4. U. Flenker, S. Hornig, E. Nolteernsting, H. Geyer, and W. Schänzer. Measurement of $^{13}C/^{12}C$-ratios to confirm misuse of endogenous steroids. In *Recent Advances in Doping Analysis (6)*, W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Edition Sport: Cologne, Germany, 1998, pp 243–256.
5. M. Ueki, M. Okano. Analysis of exogenous dehydroepiandros-

terone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Spectrom.* **13:** 2237–2243 (1999).
6. D.H. Catlin, C.K. Hatton, and S. Starcevic. Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athletes urine. *Clin. Chem.* **43:** 1280–1288 (1997).
7. D.H. van de Kerkhof, D. de Boer, J.H.H. Thijssen, and R.A.A. Maes. Evaluation of testosterone/epitestosterone ratio influential factors as determined in doping analysis. *J. Anal. Toxicol.* **24:** 102–115 (2000).
8. R. Aguilera, D.H. Catlin, M. Becchi, A. Phillips, C. Wang, R.S. Swerdloff, H.G. Pope, and C.K. Hatton. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. *J. Chromatogr. B***727:** 95–105 (1999).
9. R. Aguilera, C.K. Hatton, and D.H. Catlin. Detection of epitestosterone doping by isotope ratio mass spectrometry. *Clin. Chem.* **48:** 629–636 (2002).
10. J.-C. Mathurin, V. Herrou, E. Bourgogne, L. Pascaud, and J. de Ceaurriz. Gas chromatography–combustion–isotope ratio mass spectrometry analysis of 19-norsteroids: application to the detection of a nandrolone metabolite in urine. *J. Chromatogr. B* **759:** 267–275 (2001).
11. E. Bourgogne, V. Herrou, J.-C. Mathurin, M. Becchi, and J. de Ceaurriz. Detection of exogenous intake of natural corticosteroids by gas chromatography/combustion/ isotope ratio mass spectrometry: application to misuse in sport. *Rapid Commun. Spectrom.* **14:** 2342–2347 (2000).
12. R. Aguilera, T.E. Chapman, and D.H. Catlin. A rapid screening assay for measuring urinary androsterone and etiocholanolone $\delta^{13}C$ (‰) values by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Spectrom.* **14:** 2294–2299 (2000).
13. D.M. Jones, J.F. Carter, G. Eglinton, E.J. Jumeau, and C.S. Fenwick. Determination of $\delta^{13}C$ values of sedimentary straight chain and cyclic alcohols by gas chromatography/isotope ratio mass spectrometry. *Biol. Mass. Spec.* **20:** 641–646 (1991).
14. E. Nolteernsting, H. Geyer, U. Mareck-Engelke, W. Schänzer, and M. Donike. Standardization of the T/E determination by deuterated internal standards. In *Recent advances in Doping Analysis (2)*, M. Donike, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, Germany, 1994, pp 113–120.
15. M. Ferry, J.-C. Mathurin, M. Becchi, and J. de Ceaurriz. Influence of pregnenolone administration on IRMS analysis. In *Recent advances in Doping Analysis (8)*, M. Donike, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, Germany, 2000, pp 209–213.
16. R. Aguilera, T.E. Chapman, B. Starcevic, C.K. Hatton, and D.H. Catlin. Performance characteristics of a carbon isotope ratio method for detecting doping with testosterone based on urine diols: controls and athletes with elevated testosterone/epitestosterone ratios. *Clin. Chem.* **47:** 292–300 (2001).
17. N. Baume, L. Avois, C. Schweizer, C. Cadis, J. Dvorak, M. Cauderay, P. Mangin, and M. Saugy. [^{13}C]Nandrolone excretion in trained athletes: interindividual variability in metabolism. *Clin. Chem.* **50:** 355–364 (2004).

STEROIDS 71 (2006) 364–370

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/steroids

STEROIDS

Use of isotope ratio mass spectrometry to detect doping with oral testosterone undecanoate: Inter-individual variability of $^{13}C/^{12}C$ ratio

Norbert Baume[a], *Christophe Saudan*[a], *Aurélien Desmarchelier*[a], *Emmanuel Strahm*[a], *Pierre-Edouard Sottas*[a], *Carlo Bagutti*[b], *Michel Cauderay*[c], *Yorck Olaf Schumacher*[d], *Patrice Mangin*[a], *Martial Saugy*[a,*]

[a] *Laboratoire Suisse d'Analyse du Dopage, Institut Universitaire de Médecine Légale, Rue du Bugnon 21, 1005 Lausanne, Switzerland*
[b] *Centre Médical Vidy-Med, Rte de Chavannes 11, 1000 Lausanne 23, Switzerland*
[c] *Hôpital Riviera, Site du Samaritain, Bd. Paderewski 3, 1800 Vevey, Switzerland*
[d] *Abtlg. Sportmedizin, Medizinische Universitätsklinik Freiburg, Hugstetter Strasse 55, 79106 Freiburg, Germany*

ARTICLE INFO

Article history:
Received 22 August 2005
Received in revised form 28 October 2005
Accepted 17 November 2005
Published on line 24 January 2006

Keywords:
Testosterone undecanoate (TU)
Multiple administrations
Isotope ratio mass spectrometry (IRMS)
Doping control

ABSTRACT

The metabolic effect of multiple oral testosterone undecanoate (TU) doses over 4 weeks was assessed in seven voluntary men. The protocol was designed to detect accumulation of the substance by choosing the appropriate spot urines collections time and to study the urinary clearance of the substance after weeks of treatment. Urines were analysed by a new GC/C/isotope ratio mass spectrometry (IRMS) method to establish the $\delta^{13}C$-values of testosterone metabolites (androsterone and etiocholanolone) together with an endogenous reference compound (16(5α)-androsten-3α-ol). The significant differences in inter-individual metabolism following TU intake was illustrated by large variations in $\delta^{13}C$-values of both T metabolites (maximum $\Delta\delta^{13}C$-values = 5.5‰), as well as by very stable longitudinal T/E profiles and carbon isotopic ratios in the first hours following administration. According to T/E ratios and $\delta^{13}C$-values, the washout period after 80 mg TU intake was less than 48 h for all subjects and no accumulation phenomenon was observed upon chronic oral administration.
© 2006 Elsevier Inc. All rights reserved.

1. Introduction

Detection of doping with endogenous steroids still remains a difficult issue in sport. Monitoring indirect urinary and serum markers such as testosterone glucuronide/epitestosterone glucuronide (T/E) and testosterone glucuronide/luteinizing hormone ratios [1] or serum testosterone/17α-hydroxyprogesterone ratio [2] has been used to discriminate exogenous administration of testosterone from endogenous origin. In August 2004, a technical document from the World Anti-Doping Agency (WADA) was adopted with the need of submitting the sample to isotope ratio mass spectrometry (IRMS) analysis for determination of the $^{13}C/^{12}C$ ratio of selected steroids, if the urinary T/E ratio value is equal or greater than 4.0 as well as for altered steroid profiles [3]. If IRMS analysis does not readily indicate exogenous administration of steroids for urinary samples with T/E ratio $\geq$ 4.0, the result is reported as inconclusive and it is mandatory to conduct further longitudinal studies.

* *Corresponding author.* Tel.: +41 21 314 7332; fax: +41 21 314 7333.
E-mail address: martial.saugy@chuv.ch (M. Saugy).
0039-128X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2005.11.004

USADA 0804

Oral application of testosterone undecanoate (TU) has been found to raise serum testosterone levels in healthy and hypogonadal men [4,5]. Although testosterone is inactive on oral administration, testosterone undecanoate is absorbed from the gut via the lymphatic system and therefore avoid immediate inactivation by the liver [6,7]. Clearance of orally administered TU is fast, and hence drug testing analysis of the urinary concentration can only be performed in the first hours after administration [8,9]. In a study, it has been found that a single oral administration of 120 mg TU results in a change less than 10 h of the urinary T/E ratio for Caucasian individuals, with no significant response of used urinary markers to TU intake for one of them [10].

Although long-term effect of intramuscularly administration of T on urinary profile of androgens has been investigated [11,12], little is known about the response of urinary markers used in doping controls after chronic oral TU administrations. The purpose of the present study is to examine the urinary profile of seven subjects who received an oral dose of 80 mg TU three times a week during a period of 1 month. Carbon isotopic ratio of androsterone, etiocholanolone and 5α-androst-16-en-3α-ol (androstenol) together with T/E ratio are employed as parameters for a discussion about variability of inter-individual androgen metabolism and diagnostic criteria in doping control.

2. Material and methods

2.1. Reagents and chemicals

All chemicals were of analytical grade. All solvents were purchased from Fluka (Buchs, Switzerland). Bakerbond spe™ 500 and 1000 mg octadecyl C_{18} disposable extraction columns were obtained from JT Baker (Phillipsburg, NJ, USA). β-Glucuronidase from *Escherichia coli* in a 50% glycerol solution (pH 6.5, 140 U/ml at 37 °C) was supplied by Roche Diagnostics GmbH (Manheim, Germany). Commercial reference steroids, 17β-hydroxyandrost-4-en-3-one (testosterone, T), 17α-hydroxyandrost-4-en-3-one (epitestosterone, E), 3α-hydroxy-5α-androstan-17-one (androsterone), 3α-hydroxy-5β-androstan-17-one (etiocholanolone) and 5α-androstan-3β-ol acetate (Batch: DO125) were obtained from Steraloids Inc. (Newport, RI, USA) whereas 5α-androst-16-en-3α-ol (androstenol) was supplied by Sigma Chemical Co. (St. Louis, MO, USA). Testosterone undecanoate (Dynapharm Distribution, Meyrin, Switzerland) was analytically tested by the Pharmacy of the University Hospital (CHUV, Lausanne, Switzerland) that also prepared the pills.

2.2. Subjects and clinical protocol

The study was conducted at the Swiss Antidoping Laboratory, in Lausanne (Switzerland), after the approval of the protocol by the ethics committee of the Faculty of Medicine (University of Lausanne). Participants were seven healthy male volunteers from Caucasian origin, aged between 20 and 30 years, mainly from the Institute of Sports Sciences and Physical Education (University of Lausanne, Switzerland). All volunteers provided informed consent for the participation in the study. These students were not physically addicted to drugs and denied any participation in weightlifting or bodybuilding.

The 4-week study consisted in the oral intake of testosterone undecanoate pills (80 mg testosterone undecanoate and 115 mg mannitol) on Monday, Wednesday and Friday between 6.30 and 8 a.m. One sport urine (U1) was collected on the first day of the protocol, prior to any medication. Subsequently, spot urines were collected on day 24 before intake of the 11th pill (U2), and after 4 h (U3), 8 h (U4) and 24 h (U5). Finally, a spot urine (U6) was obtained 10 days after the last administration (day 39). A maximum standard deviation of 30 min may be considered for the collection time of all spot urines.

Four months after this protocol, the same subjects were self-administered a single dose of testosterone undecanoate (80 mg testosterone undecanoate and 115 mg mannitol) on the morning. All urine samples were collected during 24 h after intake (excretion study).

Urines were kept at 4 °C until their delivery to the laboratory. After that, each urine sample was divided into 20 ml flasks and stored at −20 °C until extraction and analyses at the laboratory.

2.3. Quantitative analysis of steroid glucuronide concentrations in urines

Quantitative analyses of urinary steroids glucuronides were performed using similar procedures of a previously reported method [10]. A six-point calibration curve was established using available reference material with the following urinary concentration range: testosterone and epitestosterone (5–4000 ng/ml, $R^2 > 0.998$), androsterone and etiocholanolone (100–10,000 ng/ml, $R^2 > 0.993$).

2.4. GC/C/IRMS analysis of urine samples

The symbol δ is the standard notation for expressing carbon isotope ratios. It is defined as parts per thousand deviation of isotopic compositions from that of Pee Dee Belemnite (PDB), and is calculated according to:

$$\delta^{13}C\,(‰) = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 1000$$

The urine sample (10 ml) was centrifugated in glass tubes at 2500 rpm for 5 min and was applied onto a C_{18} column (500 mg). The conjugated steroids were eluted with methanol (8 ml) and the eluate evaporated to dryness. To hydrolyse the conjugated steroids, the residue was dissolved in 1 ml of 0.2 M phosphate buffer (pH 7.0) and 50 μl of β-glucuronidase was added as supplied. After incubation at 50 °C during 1 h in a thermostated water bath, the deconjugated steroids were extracted on C_{18} column (1000 mg) by washing consecutively with 6 ml acetonitrile:H_2O (20:80, v/v), 6 ml acetonitrile:H_2O (35:65, v/v) and 6 ml acetonitrile:H_2O (50:50, v/v). The fraction F1 containing androsterone and etiocholanolone was obtained by elution with 3 × 6 ml acetonitrile:H_2O (75:25, v/v) and androstenol was collected by elution with 2 × 4.5 ml of acetonitrile:CH_3OH (75:25, v/v) and 2 × 4.5 ml of acetonitrile (fraction F2). The eluates were evaporated to dryness. Acetylation was carried out in 50 μl of pyridine at 60 °C for 1 h

USADA 0805

incubation with 50 μl of acetic anhydride and 50 μl of acetic anhydride/acetic acid (1:1) for fractions F1 and F2, respectively. The derivatized steroids of fractions F1 and F2 were spiked with 50 and 10 μl of an internal standard (IS, 5α-androstan-3β-ol acetate, 0.20 mg/ml in methanol), respectively. Then both fractions F1 and F2 were evaporated to dryness and finally dissolved in 200 and 40 μl of cyclohexane, respectively.

The carbon isotope measurements were performed with the instruments and conditions described elsewhere [13]. For the analysis of both fractions F1 and F2, the oven temperature was increased from 80 °C (1 min) to 270 °C (8.3 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. The fractions were injected (2 μl) in splitless mode. Possible isotopic discrimination of the steroids during the separation and analysis procedure has been tested by use of 3-year-old boy's urines (6 ml) spiked with 10 μl solution of the steroids of interest (1 mg/ml in methanol). After extraction, we found that the isotopic ratio values of the spiked steroids in urine were very similar to standard values ($\Delta\delta^{13}C < 0.70‰$). For the IRMS analysis of urine samples, we used the $\delta^{13}C$-value of 5α-androstan-3β-ol acetate (IS) as criteria of suitability ($\delta^{13}C = -30.56‰$, S.D. = 0.20‰, $n = 54$). If the $\delta^{13}C$-value of 5α-androstan-3β-ol acetate was greater than the -30.56 ± 2S.D.‰ limit, the assay was repeated. Each batch included positive (QC-Pos) and negative (QC-Neg) quality controls and the measurements were performed in the linear range of the IRMS. QC-Neg is a urine of a Caucasian male (age = 24 years, weight = 65 kg) and QC-Pos is an excretion study (pooled urines over 10 h) of 40 mg testosterone undecanoate (Panteston®, Organon SA, France) of the same individual. The within-assay precision was determined by preparing six aliquots of QC-Neg and QC-Pos in the same batch and injecting each once. The between-assay precision was determined by extracting 13 aliquots of QC-Neg and QC-Pos over 5 months. For the within-assay precision the overall means and S.D.s for etiocholanolone, androsterone and androstenol in the QC-Neg were −23.7 (0.2), −24.2 (0.2) and −23.9 (0.2)‰, respectively, whereas in the QC-Pos, −28.7 (0.3), −28.9 (0.2) and −23.6 (0.3)‰ were obtained. For the between-assay precision the overall means and S.D.s for etiocholanolone, androsterone and androstenol in the QC-Neg were −23.9 (0.5), −24.0 (0.5) and −23.5 (0.3)‰, respectively. For the QC-Pos, means and S.D.s of −28.2 (0.6), −28.2 (0.4) and −23.2 (0.5)‰ were obtained.

2.5. *Statistical analysis*

The non-parametric one-way analysis of variance (Kruskal–Wallis test) was used for statistical analysis of ^{13}C-values and T/E ratio as a function of time. Statistical analyses were performed with MATLAB (Statistics Toolbox, Version 3.0). Significant increase above the basal levels with $P < 0.05$ was considered.

3. Results and discussion

3.1. *Time-profile of T/E ratio*

As shown in Fig. 1, T/E ratios of seven Caucasian individuals have basal values in the range of 0.06–5.09. The values remain very similar for urine sample U2 collected after 3 weeks of treatment and 48 h after last TU intake ($P > 0.5$). In the course of the study, the T/E ratios show important inter-individual variability upon ingestion of the substance. Excepted for S1, the values of all subjects in samples collected after 4 h are significantly higher than their respective basal values. Eight hours after administration of the substance (U4), this ratio is still significantly higher for subjects S2, S4, S6 and S7.

To investigate the effect of chronic administrations of TU on the pharmacokinetics of the substance, the data were compared with the values obtained upon excretion studies of a single TU dose. It may be pointed out that the excretion studies displayed in Fig. 2 confirm the pharmacokinetics characterizing each of the seven individuals. Excepted for the T/E profile of subject S1 who shows almost no variation, the excretion peak occurs at 4 h for the other subjects. The T/E ratio return to basal values at about 15 h after a single dose administration for most of the individuals. Surprisingly, the pharmacokinetics of TU in subject S4 is slower with respect to the other subjects (excretion time of about 24 h). This trend was more pronounced during the multidose medication where the T/E ratio in urine U5 was significantly higher than the basal values (Fig. 1). These findings put forth an intra-individual variability in the testosterone metabolism. This is also exemplified by the comparison of the T/E ratio of subject S3 at 4 h post-administration (T/E = 17.30) with the value (T/E = 1.23) obtained during the multidose medication after similar excretion time (U3). Likewise, concentrations of testosterone metabolites were higher at the peak excretion after a single TU intake (androsterone = 12,000 ng/ml and etiocholanolone = 7200 ng/ml) compared to the concentration values found in urine U3 (androsterone = 1185 ng/ml and etiocholanolone = 1220 ng/ml). As subject S3 has apparently a very fast clearance of the substance (<5 h), it may be expected that a shift in the excretion time will have a significant influence on the steroid concentrations. The origin of this shift of about 1 h may stem from the organism hydration state.

Throughout the study, T/E ratio failed to respond to TU oral administration for subject S1 and despite of the statistical significance, this parameter was found to vary only from 0.48 to 1.23 for subject S3. This finding was also observed in two previous controlled studies of single oral TU administration [10,14] where it was supposed that these individuals are characteristic representative of a small group of Caucasian population with a low basal urinary T/E ratio. The excretion studies after a single dose administration show that the T/E ratio of S3 reached a value of 17.3 with a rapid return to basal values during the first 5 h post-administration. For subject S1, the very stable T/E ratio profile (0.09–0.20) contrasted with large concentrations of urinary T metabolites (Fig. 2). About 2 h after administration, the concentration of androsterone and etiocholanolone glucuronides were of 50,000 and 8100 ng/ml, respectively. In view of these data, both subjects differ from the five others and have likely a metabolism that converts more rapidly testosterone to downstream metabolites together with high activity of UDP-glucuronosyl transferase isoforms that conjugate androgens [15,16].

USADA 0806

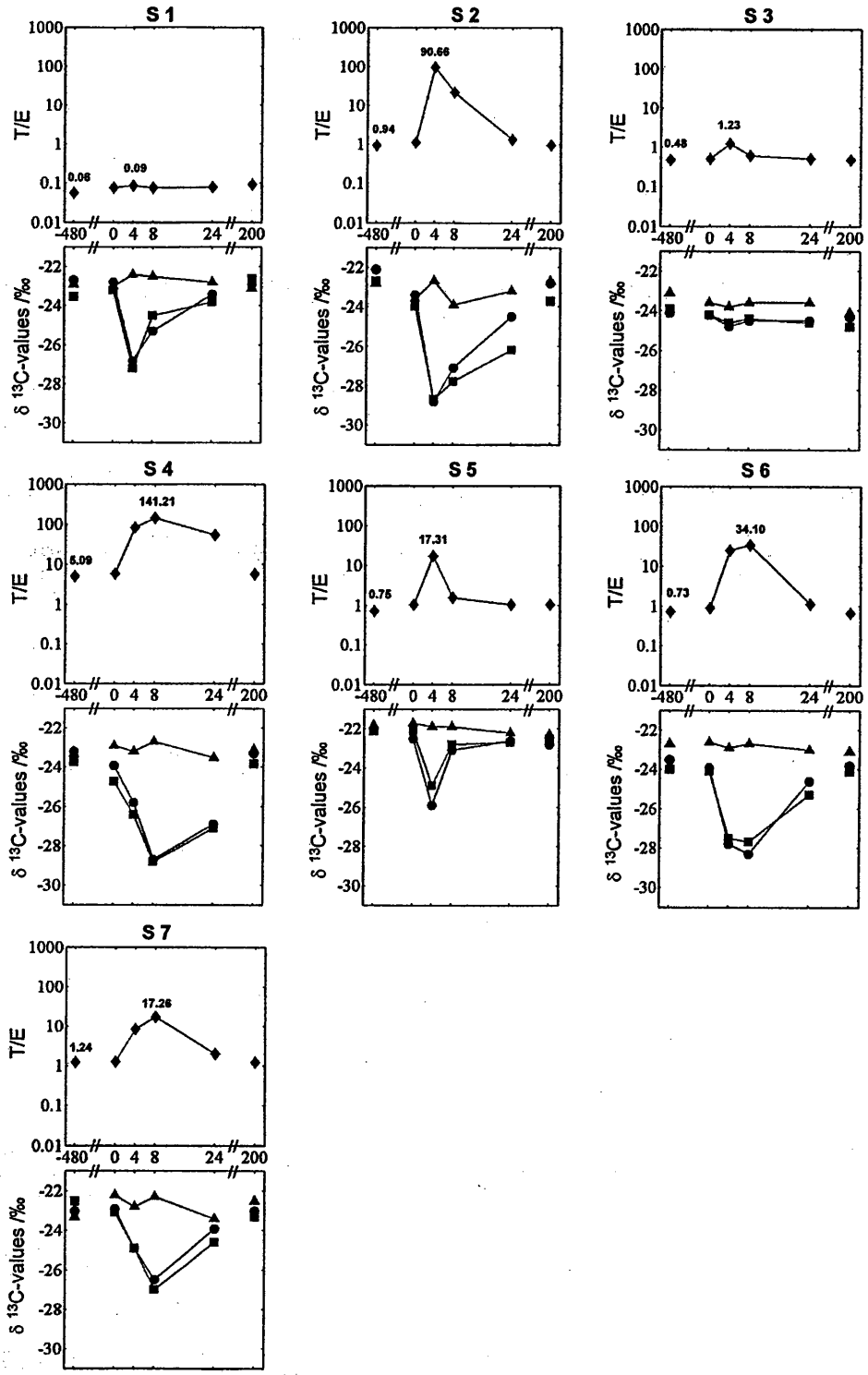


Fig. 1 – Time course in hours of urinary T/E ratio (upper panels, ♦) and δ^{13}C-values of androsterone (●), etiocholanolone (■) and androstenol (▲) of seven subjects (S1–S7) throughout the protocol study. The two x-axis breaks are shown by paired oblique lines.

3.2. *Time profile of δ^{13}C-values*

Several methods have been published for the differentiation between the exogenous and endogenous origin of steroids in urine samples [17–19]. The method described in the paper allows the detection of exogenous T in urine by comparison of δ^{13}C-values of androsterone and etiocholanolone with an endogenous reference compound (androstenol). Fig. 3 illustrates typical GC/C/IRMS traces of *m/z* 44 for fractions F1 and F2 containing T metabolites and the endogenous reference compound, respectively. We found that S.D.s on the δ^{13}C-values for each compound measured with QC-Neg and

USADA 0807

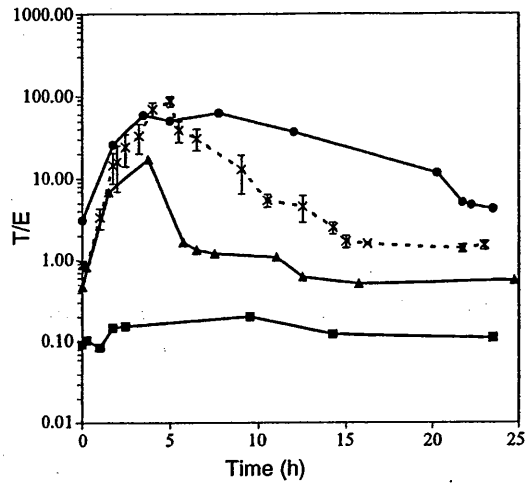


Fig. 2 – Urinary T/E ratio of subject S1 (■), S3 (▲), S4 (●) and mean values (×) (±S.E.M.) of S2, S5, S6 and S7 following a single oral dose of TU (excretion study).

QC-Pos were of about 0.2‰ in the within-assay precision and slightly higher in the case of the between-assay precision (see Section 2).

Testosterone acetate [20] prepared from TU administered in this study gave a δ^{13}C-value of −30.69 (S.D. = 0.34‰, n = 5). After correction for the negative shift of the δ^{13}C-value due to formation of the acetate [13], we obtain a δ^{13}C-value of −28.80‰ for testosterone. If no natural isotopic fractionation toward depletion in ^{13}C occurs during the in vivo metabolism of testosterone [21], maximum δ^{13}C-values of −28.80‰ may be expected for androsterone and etiocholanolone at the maximum excretion rate [17].

As depicted in Fig. 1, baseline δ^{13}C-values of steroids in urine U1 are ranging from −22.0 (S5) to −24.0‰ (S6), and thus administration of exogenous testosterone used in this study may potentially result in significant differences in the δ^{13}C-values ($\Delta\delta^{13}$C-values: 4.8–6.8‰). Similar to what found for T/E ratios, the δ^{13}C-values of the steroids in urine sample U2 are not significantly different from those determined in U1 ($P > 0.5$). Accumulation phenomenon caused by body storage and slow elimination in the urine was therefore not observed in all subjects. Conversely, the data tend to show that the washout period is less than 48 h for all subjects. Samples collected 0, 4, 8 and 24 h after intake of a pill may provide information on individual variability of TU clearance in the urines. The time course of isotopic values of androsterone, etiocholanolone and androstenol in urines U2, U3, U4 and U5 for all subjects are shown in Fig. 1. As observed in QC-Pos urine sample, the δ^{13}C-values of androstenol do not display significant changes upon oral TU administration, while δ^{13}C-values of both T metabolites undergo large variations. As mentioned above, the results obtained with some individuals (S2, S4 and S6) tend to demonstrate that androsterone and etiocholanolone contained in urines U3 and U4 originates almost completely from the conversion of TU, with limited contribution of endogenous steroids metabolism. Global statistical analysis shows significance ($P < 0.02$) with respect to basal values for androsterone and etiocholanolone in urines U3 and U4. However, it should be noted that for urine U5 of subjects S2 and S4, the δ^{13}C-values differ from 1 to 3‰ compared to those found before TU intake. As observed on the urinary T/E profiles, these findings reflect the important inter-individual variability in the metabolic clearance of TU. To underline this variability, it is worth mentioning the data obtained with S3, which show no significant variation in δ^{13}C-values of the steroids of interest (Fig. 1). As discussed below, the stability of the δ^{13}C-values of this subject during the multidose medication study may be explained by fast metabolization and excretion of the substance. Nevertheless, we observed a small shift in the excretion rate of TU in this protocol with respect to single excretion studies. Therefore, it may be expected that the spot urine collected 4 h after a single dose excretion would have lead to more negative δ^{13}C-values for testosterone metabolites.

Spot urine U6 has been collected at the end of the treatment, i.e. 10 days after intake of the last pill. The δ^{13}C-values in urine U6 are similar to what found initially (U1) and confirm the observation found with urine U2. Throughout the course of the excretion of all individuals, high correlation coefficients were found between absolute carbon isotopic data and the urinary concentration profiles of androsterone ($r = 0.69$; $P < 1 \times 10^{-5}$) and etiocholanolone ($r = 0.51$; $P < 1 \times 10^{-3}$).

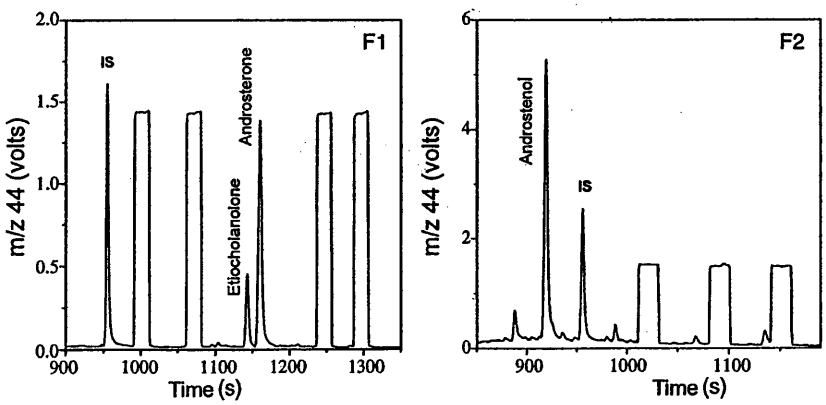


Fig. 3 – Typical GC-C-IRMS *m/z* 44 mass chromatograms of fractions F1 (androsterone and etiocholanolone acetates) and F2 (androstenol acetate) spiked with an internal standard (IS, 5α-androstan-3β-ol acetate). The square-topped peaks represent pulses of CO_2 reference gas.

USADA 0808

3.3. *Implications in anti-doping analyses*

According to the technical document of the WADA Laboratory Committee, an athlete would be reported as consistent with the administration of a steroid when the $^{13}C/^{12}C$-value measured for the metabolites differs significantly, i.e. by 3.0‰ or more from that of the urinary reference steroid chosen [3]. The samples submitted to IRMS analysis are those with T/E ratio ≥ 4 or with abnormal steroids glucuronide concentrations: T and E > 200 ng ml^{-1}, androsterone and etiocholanolone > 10,000 ng ml^{-1} and DHEA > 100 ng ml^{-1} [3].

In this study, all individuals have basal T/E values ranging from 0.06 to 1.25, excepted for subject S4 in whom T/E is of 5.09 (Fig. 1). It has to be noticed that there is a high and significant correlation between the basal and the maximum T/E values ($r = 0.84$, $P = 0.0086$). Interestingly, the treatment with TU is not accompanied with an increase of T/E values above 4.0 in urines of subjects S1 and S3. Similarly, the steroid concentrations for these individuals were well below the concentration cut-offs mentioned previously (data not shown), and consequently the samples would not have been subjected to IRMS analysis in an anti-doping control, though the $^{13}C/^{12}C$-values measured for androsterone and etiocholanolone differ by more 3.0‰ from that of androstenol in urine U3 of subject S1 (Fig. 1). Another interesting feature of this study is depicted by T/E profile of subject S7 which displays significant variations 4 h after TU intake compared to basal value (Fig. 1), whereas IRMS data of U3 are not conclusive ($\Delta\delta^{13}C$-values = 2.0‰).

Our study shows that about 60% of the samples collected during the first hours after oral intake of 80 mg TU (7 urines U3 and U4) would have been reported as indicative of administration of an endogenous steroid. If complementary specific analyses would have been conducted [10,22], a misuse of TU for both subjects with stable steroid profiles could have been revealed.

4. Conclusion

Chronical oral TU administration throughout a month do not influence notably the urinary steroid profiles in the seven volunteers. Nevertheless, most of the urines taken 4 and 8 h after administration of the TU showed significant and variable changes in the T/E ratio and in carbon isotopic ratios of androsterone and etiocholanolone. In some cases, it appears that the $\delta^{13}C$-values of testosterone metabolites are more valuable urinary markers than the common markers derived from steroid concentrations.

An interesting feature of this study is displayed by two of the seven subjects for whom the excretion occurs in the first hours after administration. Thus, it is conceivable that testing of these individuals in doping control would lead likely to negative results when using common urinary markers. In order to increase the sensitivity of TU detection, further investigations on this group of individuals are needed for identification of specific biomarkers of this class of doping agent.

Acknowledgements

This clinical study was entirely supported by grants from the Swiss Federal Office for Sport (FOSPO). The IRMS development and measurements were financially supported by the Fédération Internationale de Football Association (FIFA) Medical Assessment and Research Center (FIFA/F-MARC, Zürich, Switzerland).

REFERENCES

[1] Perry PJ, MacIndoe JH, Yates WR, Scott SD, Holman TL. Detection of anabolic steroid administration: ratio of urinary testosterone to epitestosterone vs the ratio of urinary testosterone to luteinizing hormone. Clin Chem 1997;43:731–5.

[2] Carlström K, Palonek E, Garle M, Oftebro H, Stanghelle J, Björkhem I. Detection of testosterone administration by increased ratio between serum concentrations of testosterone and 17α-hydroxyprogesterone. Clin Chem 1992;38:1779–84.

[3] WADA technical document: reporting and evaluation guidance for testosterone, epitestosterone, T/E ratio and other endogenous steroids; 2004. http://www.wada-ama.org.

[4] Davidson DW, O'Carroll R, Bancroft J. Increasing circulating androgens with oral testosterone undecanoate in eugonadal men. J Steroids Biochem Mol Biol 1987;26:713–5.

[5] Giagulli VA, Verdonck L, Deslypere JP, Giorgino R, Vermeulen A. Comparison of plasma androgen glucuronide levels after percutaneous or peroral androgen treatment in men: evidence for important splanchnic contribution to plasma 17β-hydroxyandrogen glucuronides. J Clin Endocrinol Metab 1993;76:429–31.

[6] Nieschlag E, Mauss J, Coert A, Kićovic P. Plasma androgen levels in men after oral administration of testosterone or testosterone undecaonate. Acta Endocrinol 1975;79:366–74.

[7] Coert A, Geelen J, de Visser J, van der Vies J. The pharmacology and metabolism of testosterone undecanoate (TU), a new orally active androgen. Acta Endocrinol 1975;79:789–800.

[8] Wright F, Lafarge JP, Antréassian J, Lagoguey M, Pérès G. Long term study of steroid and peptidic hormones in the plasma of healthy young men under controlled testosterone undecanoate therapy. In: Hemmersbach P, Birkeland KI, editors. Blood samples for doping control. Pensumtjeneste: Oslo; 1994. p. 65–74.

[9] Maître A, Saudan C, Mangin P, Saugy M. Urinary analysis of four testosterone metabolites and pregnanediol by gas chromatography/combustion/isotope ratio mass spectrometry after oral administrations of testosterone. J Anal Toxicol 2004;28:426–31.

[10] Peng SH, Segura J, Farré M, González JC, de la Torre X. Plasma and urinary markers of oral testosterone undecanoate misuse. Steroids 2002;67:39–50.

[11] Palonek E, Gottlieb C, Garle M, Björkhem I, Carlström K. Serum and urinary markers of exogenous testosterone administration. J Steroids Biochem Mol Biol 1995;55:121–7.

[12] Dehennin L, Matsumoto AM. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. J Steroids Biochem Mol Biol 1993;44:179–89.

[13] Saudan C, Baume N, Mangin P, Saugy M. Urinary analysis of 16(5α)-androsten-3α-ol by gas

USADA 0809

chromatography/combustion/isotope ratio mass spectrometry: implications in anti-doping analysis. J Chromatogr B 2004;810:157–64.
[14] Dehennin L, Peres G. Plasma and urinary markers of oral testosterone misuse by healthy men in presence of masking epitestosterone administration. Int J Sports Med 1996;17:315–9.
[15] Coffman BL, King CD, Rios GR, Tephly TR. The glucuronidation of opioids, other xenobiotics, and androgens by human UGT2B7Y(268) and UGT2B7H(268). Drug Metab Disp 1998;26:73–7.
[16] de Wildt SN, Kearns GL, Leeder JS, van der Anker JN. Glucuronidation in humans: pharmacogenetic and developmental aspect. Clin Pharmacokinet 1999;36:439–52.
[17] Shackleton CHL, Phillips A, Chang T, Li Y. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids 1997;62:379–87.
[18] Balizs G, Jainz A, Horvatovich P. Investigation of the feeding effect on the $^{13}C/^{12}C$ isotope ratio of the hormones in bovine urine using gas chromatography/combustion/isotope ratio mass spectrometry. J Chromatogr A 2005;1067:323–30.
[19] Aguilera R, Chapman TE, Starcevic B, Hatton CK, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with testosterone based on urine diols: controls and athletes with elevated testosterone/epitestosterone ratios. Clin Chem 2001;47:292–300.
[20] de la Torre X, González JC, Pichini S, Pascual JA, Segura J. $^{13}C/^{12}C$ isotope ratio MS analysis of testosterone, in chemicals and pharmaceutical preparations. J Pharm Biomed Anal 2001;24:645–50.
[21] Brenna JT. Natural intramolecular isotope measurements in physiology: elements of the case for an effort toward high-precision position-specific isotope analysis. Rapid Commun Spectrom 2001;15:1252–62.
[22] Shackleton CHL, Chuang H, Kim J, de la Torre X, Segura J. Electrospray mass spectrometry of testosterone esters: potential for use in doping control. Steroids 1997;62:523–9.

USADA 0810

Clinical Chemistry 47:2
292–300 (2001)

Drug Monitoring and Toxicology

Performance Characteristics of a Carbon Isotope Ratio Method for Detecting Doping with Testosterone Based on Urine Diols: Controls and Athletes with Elevated Testosterone/Epitestosterone Ratios

RODRIGO AGUILERA,[1*] THOMAS E. CHAPMAN,[1] BORISLAV STARCEVIC,[1] CAROLINE K. HATTON,[1] and DON H. CATLIN[1,2]

Background: **Carbon isotope ratio methods are used in doping control to determine whether urinary steroids are endogenous or pharmaceutical.**

Methods: **Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) was used to determine the $\delta^{13}C$ values for 5β-androstane-3α,17β-diyl diacetate (5βA), 5α-androstane-3α,17β-diyl diacetate (5αA), and 5β-pregnane-3α,20α-diyl diacetate (5βP) in a control group of 73 healthy males and 6 athletes with testosterone/epitestosterone ratios (T/E) >6.**

Results: **The within-assay precision SDs for 5βA, 5αA, and 5βP were ± 0.27‰, ± 0.38‰, and ± 0.28‰, respectively. The between-assay precision SDs ranged from ± 0.40‰ to ± 0.52‰. The system suitability and batch acceptance scheme is based on SDs. For the control group, the mean $\delta^{13}C$ (SD) values were −25.69‰ (± 0.92‰), −26.35‰ (± 0.68‰), and −24.26‰ (± 0.70‰), for 5βA, 5αA, and 5βP, respectively. 5βP was greater than 5βA and 5αA ($P < 0.01$), and 5βA was greater than 5αA ($P < 0.01$). The means − 3 SD were −28.46‰, −28.39‰, and −26.37‰ for 5βA, 5αA, and 5βP, respectively. The maximum difference between 5βP and 5βA was 3.2‰, and the maximum 5βA/5βP was 1.13. Three athletes with chronically elevated T/Es had $\delta^{13}C$ values consistent with testosterone administration and three did not.**

Conclusions: **This GC-C-IRMS assay of urine diols has low within- and between-assay SDs; therefore, analysis of one urine sample suffices for doping control. The means, SDs, ±3 SDs, and ranges of $\delta^{13}C$ values in a control group are established. In comparison, testosterone users have low 5βA and 5αA, large differences between 5βA or 5αA and 5βP, and high 5βA/5βP and 5αA/5βP ratios.**

© 2001 American Association for Clinical Chemistry

A critical issue in doping control is establishing the origin of testosterone and other steroids typically found in human urine. If the origin can be shown to be pharmaceutical, as opposed to endogenous, a doping offense has occurred. Androgens for pharmaceutical use are not synthesized de novo; they are obtained by semisynthesis from starting materials such as diosgenin and stigmasterol, which are derived from plants *(1)*. Furthermore, these compounds have less ^{13}C than their endogenous homologs *(2, 3)*; therefore, urinary steroids with a low $^{13}C/^{12}C$ ratio are likely to have originated from pharmaceutical sources. This thesis has led to the use of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)[3] in doping control because the method determines the $\delta^{13}C$ value of steroids extracted from urine, based on the equation:

[1] UCLA Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology and [2] Department of Medicine, University of California at Los Angeles, Los Angeles, CA 90025.

*Address correspondence to this author at: UCLA Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology, University of California at Los Angeles, 2122 Granville Ave., Los Angeles, CA 90025-6106. Fax 310-206-9077; e-mail rodrigoa@ucla.edu.

Received June 7, 2000; accepted November 22, 2000.

[3] Nonstandard abbreviations: GC-C-IRMS, gas chromatography-combustion-isotope ratio mass spectrometry; T/E, testosterone/epitestosterone ratio; 5βA, $\delta^{13}C$ value for 5β-androstane-3α,17β-diyl diacetate; 5αA, $\delta^{13}C$ value for 5α-androstane-3α,17β-diyl diacetate; 5βP, $\delta^{13}C$ value for 5β-pregnane-3α,20α-diyl diacetate; and QC, quality control.

USADA 0811

$$\delta^{13}C[‰] = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 1000$$

where $^{13}C/^{12}C$ refers to the isotope ratio in the sample or an international standard (a reference gas calibrated relative to Pee Dee Belemnite). Accordingly, for several years, GC-C-IRMS has been applied to detect the use of exogenous steroids such as testosterone *(3, 4)*, dihydrotestosterone *(5, 6)*, and dehydroepiandrosterone *(6, 7)* by athletes.

Testosterone administration increases the ratio of urine testosterone to epitestosterone (T/E). This finding led to the use of T/E as a screening test for testosterone administration *(8)*. If the ratio exceeds 6, laboratories accredited by the International Olympic Committee report the result to the sport authority *(9)*. It is known, however, that factors other than testosterone administration may also increase the T/E ratios *(10–12)*. Furthermore, testosterone administration does not always lead to an elevated[4] T/E ratio *(3, 13)*. These factors complicate the interpretation of elevated T/E ratios and limit the significance of T/E ratios <6. Determining the T/E time profile of an individual is useful, but it requires consideration of the results of past tests or the collection of additional samples. Our previous work demonstrated that the measurement of $\delta^{13}C$ values of testosterone and its metabolites can detect testosterone use *(4, 14, 15)*. Others have reported that $\delta^{13}C$ values may be abnormally low even in samples with T/E ratios <6 *(3)*.

With the introduction of new methods, it is essential to fully characterize the assay and to establish the values in the control group. This is particularly true with $\delta^{13}C$ measurements when applied to drug control because the method is inherently difficult, the analytical results are likely to be litigated, and it has been suggested *(3, 5)* that diet or ethnicity may influence the $^{13}C/^{12}C$ ratio of urine steroids. Accordingly, herein we characterize the $\delta^{13}C$ measurements of the diacetates of 5β- and 5α-androstane-3α,17β-diol (5βA and 5αA, respectively), and 5β-pregnane-3α,20α-diol (5βP) with respect to precision, linearity, system suitability, and batch acceptance. In addition, we determined $\delta^{13}C$ values for these steroids in a control group of 73 healthy male subjects from four different ethnic groups. Finally, data gathered from the control group were used to interpret $\delta^{13}C$ values obtained on athletes with elevated T/E ratios and to discuss diagnostic criteria for doping.

Materials and Methods

STEROID CALIBRATION SOLUTION

A steroid calibration solution was prepared that contained 25 mg/L 5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol, and 5β-pregnane-3α,20α-diol, all as diacetates (Steraloids, Inc.).

CONTROL GROUP

The control group consisted of 74 male medical students (age range, 19–29 years) from the University of California at Los Angeles. Body weights were 45–110 kg. The students had the option to give a medical and drug history and to describe their ethnicity. No student declared taking steroids or reported any chronic disease. The ethnicities were "Asian" (n = 16), "African American" (n = 9), "Caucasian" (n = 25), "Hispanic" (n = 13), "other" (n = 7), and "unknown" (n = 4). The students collected 24-h urine samples, and 50-mL aliquots were stored at 4 °C. The study was conducted under the guidance of the Human Subject Protection Committee of the University of California at Los Angeles.

ATHLETES WITH ELEVATED T/E RATIOS

Of the large number of anonymous urine samples that our laboratory analyzes for sport drug control programs in the US, some have a urine T/E ratio >6. In such cases, the sport organization typically collects additional samples to track the urine T/E over weeks or months. We selected some of these urine samples for GC-C-IRMS analysis based on the availability of at least two samples per person, sufficient volume to perform the analysis, and permission from the sport organization.

QUALITY-CONTROL URINE SAMPLES

Quality-control urines (QC-H and QC-L) were obtained from two subjects known not to be using testosterone or any substance likely to alter the $\delta^{13}C$ values of 5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol, and 5β-pregnane-3α,20α-diol (measured as diacetates). Each subject donated one 8-h urine, which was aliquoted into 10-mL cryogenic tubes and stored at −20 °C until analysis.

URINARY STEROID CONCENTRATIONS AND SAMPLE PREPARATION FOR GC-C-IRMS

The T/E ratio was estimated by GC-MS as described previously *(16)*. The concentrations of 5β- and 5α-androstane-3α,17β-diol were estimated from a QC sample prepared by adding to steroid-free urine 5β- and 5α-androstane-3α,17β-diol. [16,16,17-^{2}H]-testosterone (CDN Isotopes) was used as an internal standard. The 5β-pregnane-3α,20α-diol was not quantified. The sample preparation methods for GC-C-IRMS analysis and the instrument conditions have been described *(15)*. The volume of urine extracted for GC-C-IRMS was 10 mL.

GC-C-IRMS DETERMINATION OF 5αA, 5βA, AND 5βP

The GC-C-IRMS analyses were conducted on a Finnigan Delta Plus Isotope Ratio Mass Spectrometer. The IRMS was connected to a Hewlett Packard Model 6890 gas chromatograph via a Finnigan Combustion III interface. The GC was equipped with an HP-50+ capillary column [30 m × 0.25 mm (i.d.); 0.15 μm film thickness] and a Finnigan A200S autosampler. The combustion oven was

[4] The word "elevated" is used to refer to a result that is above the administrative cutoff.

USADA 0812

oxidized by back-flushing with oxygen for 1 h every 30 samples. The oxidation reactor in the combustion oven was replaced every 500 samples. The injection volume was 1 μL. The recoveries for 5β- and 5α-androstane-3α,17β-diol were 85% and 86%, respectively.

GC-C-IRMS RESPONSE LINEARITY

The linearity of the GC-C-IRMS response was determined by preparing seven solutions containing all three analytes at 2.5, 5, 10, 25, 50, 100, and 150 mg/L, respectively. One microliter of each solution was injected three times on 1 day, and the three $\delta^{13}C$ values for each compound were averaged.

PRECISION STUDIES

Instrument precision for 5βP, 5βA, and 5αA was determined by extracting one aliquot of each QC urine and injecting each extract four times in succession. The within-day precision was determined by extracting 20 aliquots of QC-H in the same batch and injecting each one once. The between-assay precision was determined by extracting one aliquot of QC-H and QC-L per day for 16 days, spanning 15 months, and injecting each aliquot once.

DAILY SYSTEM SUITABILITY TEST

To establish a tolerance range, the steroid calibrator was injected five times on each of 5 different days over 2 weeks (n = 25), and the mean $\delta^{13}C$ values and SDs were calculated for each steroid. Each day, before urine sample analysis, the system suitability was assessed by injecting the calibrator three times and calculating the mean $\delta^{13}C$ values. The system was considered suitable for batch analyses if the mean $\delta^{13}C$ values of at least two of the three steroids were within ± 2 SD of the means described above. If the system suitability test failed, maintenance was performed on the gas chromatograph injection port, the GC column and/or the oxidation reactor were replaced, and the calibrator was re-injected. Data acceptance criteria included absence of peak tailing, retention times of the steroids within ± 1% of established values, and a minimum 0.8 V response at *m/z* 44 for each steroid.

CRITERIA FOR BATCH ACCEPTANCE

To establish a tolerance range for the QC urines for batch data acceptance, two aliquots of each QC urine were extracted and each injected twice per day for 5 days spanning 2 weeks (n = 20). ANOVA was performed using the factors day (5), injection (2), duplicate (2), and QC (2). The mean $\delta^{13}C$ values and SDs were calculated for each of the three steroids. For batch analysis, each unknown urine sample was extracted and injected once. One aliquot of the two QC urines and one aliquot of the steroid calibrator were analyzed with each batch of samples. These three controls were injected at the beginning, in the middle, and at the end of the batch. The batch was accepted if at least six of the nine means for the three steroids were within ± 2 SD of the previously established means. The acceptance criteria were as noted above except that an instrument response of 0.3 V at *m/z* 44 was accepted.

STATISTICAL ANALYSES

The Smirnov-Grubbs method was used to test the control group for outliers; otherwise, all statistical tests utilized statistical software (SAS). The linearity of the GC-C-IRMS response was assessed by least-squares linear regression. The response was considered linear if the slope was zero at $P = 0.01$. The method of Koch and Peters *(17)* was used to determine the SDs of duplicates. The normality of the distributions of 5αA, 5βA, and 5βP, differences, and ratios was determined by the Anderson-Darling test. The values for 5αA, 5βA, and 5βP were correlated with Pearson's correlation coefficient, *r*. Two-sided paired *t*-tests were used to compare the means of 5αA, 5βA, and 5βP in the control group. The general linear model procedure was used to assess differences between the mean $\delta^{13}C$ values of the ethnic groups, and the power of the analysis was assessed by one-way ANOVA.

Results

LINEARITY OF GC-C-IRMS RESPONSE

The IRMS response was linear for 5β- and 5α-androstane-3α,17β-diyl diacetate from 2.5 to 150 mg/L (Fig. 1). However, for 5β-pregnane-3α,20α-diyl diacetate, the response was linear only between 5 and 150 mg/L. The concentrations of 5β- and 5α-androstane-3α,17β-diol in the control group were 23–430 and 25–204 μg/L, respectively. Therefore, assuming 85% recovery, the amounts of 5β- and 5α-androstane-3α,17β-diol extracted from the 10-mL urine and reconstituted in 25 μL of cyclohexane were analyzed in the linear range of the IRMS.

PRECISION OF THE GC-C-IRMS INSTRUMENT AND ASSAY

Instrument precision (SD) for 5βA in QC-H and QC-L was 0.32‰ and 0.41‰, respectively. For 5αA, it was 0.08‰

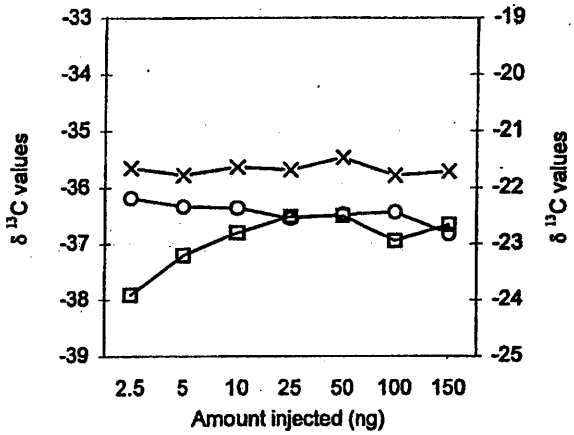


Fig. 1. Relationships between concentration of 5β-androstane-3α,17β-diyl diacetate (×), 5α-androstane-3α,17β-diyl diacetate (○), 5β-pregnane-3α,20α-diyl diacetate (□; *right axis*) and $\delta^{13}C$ values.

and 0.59‰, respectively, and for 5βP, it was 0.27‰ and 0.16‰, respectively. The descriptive statistics for the within-assay experiment on QC-H are shown in Table 1. The SDs were 0.27‰, 0.38‰, and 0.28‰ for 5βA, 5αA, and 5βP, respectively, and the CVs were ≤1.4%. The range of values was 0.9‰ for 5βA, 1.2‰ for 5βP, and 1.8‰ for 5αA. The between-assay SDs for QC-H were 0.40‰, 0.42‰, and 0.44‰ for 5βA, 5αA, and 5βP, respectively, and the CVs were ≤1.8% (Table 2). For QC-L, the values were slightly higher. The mean $\delta^{13}C$ values for the three steroids in QC-L were significantly lower than the means for QC-H.

SYSTEM SUITABILITY AND BATCH ACCEPTANCE

In the system suitability test, the SDs of 5βA, 5αA, and 5βP were 0.31‰, 0.59‰, and 0.54‰, respectively. The tolerance range for the batch acceptance data provided SDs for 5βA, 5αA, and 5βP of 0.68‰, 0.67‰, and 0.65‰ for QC-H, and 0.66‰, 0.56‰, and 0.39‰ for QC-L, respectively. Because these data consisted of duplicate analyses, we calculated the SDs of duplicates and performed an ANOVA. The SDs of duplicates were 0.24‰, 0.32‰, and 0.37‰ for 5βA, 5αA, and 5βP, respectively. These values were approximately one-half the SDs obtained for the factor injection. ANOVA revealed that QC-H and QC-L were different, and there was no difference for the factors injection, duplicate, or day.

CONTROL SUBJECTS: DESCRIPTIVE STATISTICS FOR THE CONCENTRATIONS AND $\delta^{13}C$ OF URINARY STEROIDS

The geometric means for urine testosterone, epitestosterone, and 5β- and 5α-androstane-3α,17β-diol concentrations were 20, 29, 98, and 58 μg/L, respectively. The geometric mean for urine T/E was 0.7 (arithmetic range, 0.2–5.3). The concentration of urine 5β-pregnane-3α,20α-diol was not determined. Table 3 and Fig. 2 show the descriptive statistics and the histograms for 5βA, 5αA, and 5βP. The outlier test was applied, and no data points were excluded. The distributions of 5αA and 5βP were gaussian (A^2 = 0.28, P >0.25; and A^2 = 0.21, P >0.25, respectively). The distribution of 5βA was gaussian at P = 0.01, but not gaussian at P = 0.05, A^2 = 0.85.

There were significant (P <0.0001) correlations between 5βA and 5αA (r = 0.70), between 5βA and 5βP (r = 0.67), and between 5βP and 5αA (r = 0.58). The CVs of the $\delta^{13}C$ values for the diacetates were 2.6–3.6%. The means − 3 SD for 5βA, 5αA, and 5βP were −28.46‰, −28.39‰, and −26.37‰, respectively. The mean 5αA was lower (−26.35‰) than the mean 5βA (−25.69‰; P <0.001). The Wilcoxon nonparametric two-sample test confirmed that 5αA was lower than 5βA. The means for 5βA and 5αA were lower than the mean for 5βP (both P <0.001).

Table 3 also summarizes the statistics of the differences between the means of 5βP and 5βA (5βP − 5βA) and 5βP and 5αA (5βP − 5αA), and the ratios 5βA/5βP and 5αA/5βP. The distributions of 5βP − 5βA and 5βP − 5αA were gaussian (A^2 = 0.23 and 0.19, respectively). The 5βP − 5βA differences ranged from −0.08‰ to 3.17‰, and the mean + 3 SD was 3.47‰. The 5βP − 5αA differences ranged from 0.16‰ to 3.72‰, and the mean + 3 SD was 3.99‰. The distributions of 5βA/5βP and 5αA/5βP were gaussian (A^2 = 0.19 and 0.21, respectively). The means of 5βA/5βP and 5αA/5βP were 1.06 and 1.09, respectively, and their corresponding means + 3 SD were 1.14 and 1.17. There were no differences between ethnic groups for the means of 5αA, 5βA, or 5βP (Table 4).

Table 1. Within-assay precision for 5βA, 5αA, and 5βP.

	QC-H		
	5βA	5αA	5βP
Mean, ‰ (n = 20)	−24.46	−26.83	−23.13
SD, ‰	0.27	0.38	0.28
CV, %	1.1	1.4	1.2
Minimum, ‰	−24.90	−27.81	−23.62
Maximum, ‰	−23.96	−26.05	−22.47
Range, ‰	0.9	1.8	1.2

$\delta^{13}C$ VALUES IN ATHLETES WITH ELEVATED T/E RATIOS

The urine concentrations of testosterone and epitestosterone, the T/E ratios, and the $\delta^{13}C$ values for the six male athletes with urine T/E ratios >6 are summarized in Table 5. Of the six, athletes 1 and 2 were known to be taking testosterone for medical reasons. According to the sport organizations ordering the tests, athletes 3–6 denied taking any substance known to influence the T/E ratio. The number of samples per athlete ranged from three to eight. The T/E ratio of all samples was >6 for four of the six athletes. Athlete 4 had one T/E ratio of 5.0, and athlete 5 had one T/E ratio of 5.1; otherwise, all T/E ratios were >6.

Table 5 reveals that for athletes 1–3, all 5βA and 5αA values were lower than the mean − 3 SD values of the controls in Table 3, whereas the 5βP values were all close to the mean 5βP value of the controls (−24.26‰). The cells in Table 5 that are outside the ± 3 SD values of Table 4 are outlined or shaded to facilitate comparisons. The combination of 5βP values that are near the mean and low 5βA and 5αA values produce large differences in 5βP −

Table 2. Between-assay precision for 5βA, 5αA, and 5βP.

	QC-H			QC-L		
	5βA	5αA	5βP	5βA	5αA	5βP
Mean, ‰	−24.72	−26.11	−24.00	−26.99	−27.57	−25.97
SD, ‰	0.40	0.42	0.44	0.50	0.47	0.52
CV, %	1.6	1.6	1.8	1.8	1.7	2.0
Minimum, ‰	−25.43	−27.19	−24.47	−27.65	−28.20	−26.86
Maximum, ‰	−24.06	−25.44	−22.89	−25.69	−26.55	−24.85
Range, ‰	1.4	1.8	1.6	2.0	1.7	2.0
n	15	14	16	15	14	16

Table 3. Control group of 73 subjects: Descriptive statistics for $\delta^{13}C$, differences between the means, and ratios of the means.

	$\delta^{13}C$, ‰			Difference, ‰		Ratio	
	5βA	5αA[a]	5βP[b]	5βP − 5βA	5βP − 5αA	5βA/5βP	5αA/5βP
Mean	−25.69	−26.35	−24.26	1.43	2.09	1.06	1.09
SD	0.92	0.68	0.70	0.68	0.63	0.028	0.027
CV, %	3.6	2.6	2.9			2.7	2.5
Mean + 3 SD	−22.92	−24.31	−22.15	3.47	3.99	1.14	1.17
Mean − 3 SD	−28.46	−28.39	−26.37	−0.62	0.18	0.97	1.00
Maximum	−23.90	−24.55	−22.92	3.17	3.72	1.13	1.16
Minimum	−27.82	−27.89	−25.49	−0.08	0.16	1.00	1.01
Max − Min	3.9	3.3	2.6				

[a] Mean significantly different from 5βA.
[b] Mean significantly different from 5βA and 5αA.

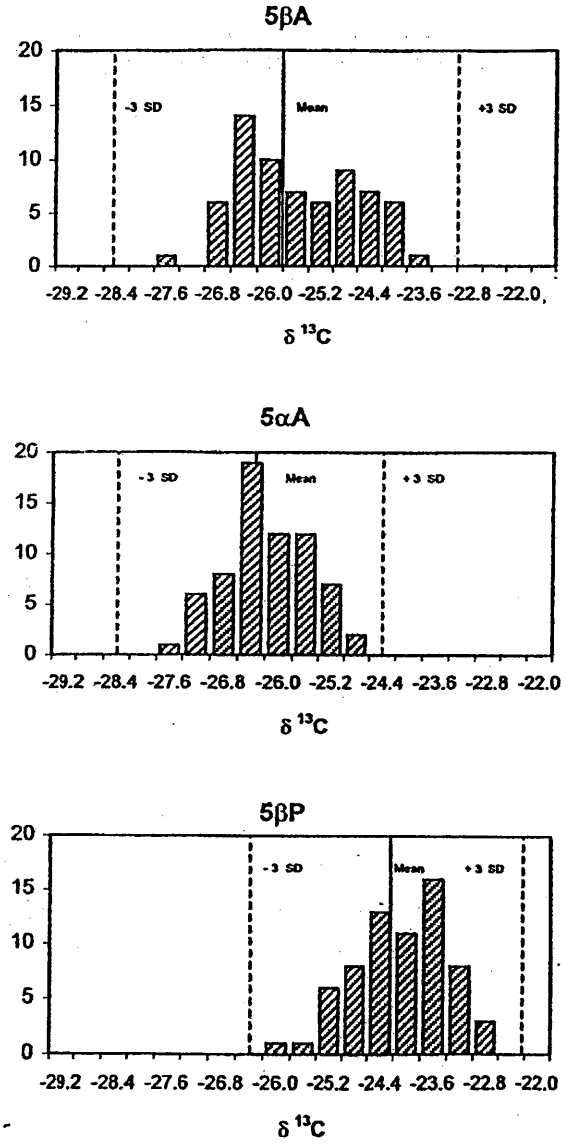


Fig. 2. Histograms of $\delta^{13}C$ values for 5βA (*top*), 5αA (*middle*), and 5βP (*bottom*) for a control group of 73 healthy males.

5βA and 5βP − 5αA, and large 5βA/5βP and 5αA/5βP ratios. For athletes 1–3, all differences and ratios were outside the + 3 SD range. The means of athletes 1–3 and 4–6 and their *z*-scores are presented at the bottom of Table 5. Another aspect of athletes 1–3 was that all 5αA values were lower than the 5βA values for the same urine: the means were −29.84‰ for 5βA and −31.83‰ for 5αA ($P = 0.04$). The means of 5βP − 5βA (5.9‰; *z*-score = 6.5) and of 5βP − 5αA (7.9‰; *z*-score = 9.1) were substantially greater than the + 3 SD values determined for the control group. Similarly, the mean 5βA/5βP ratio was 1.25 (*z*-score = 6.6), and the mean 5αA/5βP ratio was 1.33 (*z*-score = 8.9).

To compare the testosterone concentrations in Table 5 to the general population of athletes that we routinely test, we determined the log mean (3.22 μg/L) and log SD (1.19) of the distribution of the latest 11 938 male urine samples tested in our laboratory. The urine testosterone concentrations in subjects 1 and 2, the two permitted testosterone users, ranged from a low of 2.3 μg/L on the first urine ever collected to 186 μg/L (*z*-score = 1.5, in log

Table 4. Control group: Descriptive statistics for 5βA, 5αA, and 5βP by ethnicity.

Steroid	Ethnicity	n	Mean, ‰	SD, ‰	CV, %
5βA	Cauc.[a]	25	−25.74	0.95	3.7
	Asian	15	−26.17	0.66	2.5
	Hispanic	13	−25.02	1.03	4.1
	A.A.	9	−25.56	0.82	3.2
5αA	Cauc.	25	−26.43	0.79	3.0
	Asian	15	−26.60	0.49	1.8
	Hispanic	13	−26.06	0.73	2.8
	A.A.	9	−26.25	0.79	3.0
5βP	Cauc.	25	−24.34	0.70	2.9
	Asian	15	−24.19	0.68	2.8
	Hispanic	13	−24.14	0.92	3.8
	A.A.	9	−24.22	0.70	2.9

[a] Cauc., Caucasian; A.A., African American.

Table 5. Concentrations of testosterone and epitestosterone; T/E ratios; $\delta^{13}C$ values for 5βA, 5αA, and 5βP; difference scores between 5βP and testosterone metabolites (5βA and 5αA); and ratios of metabolites to 5βP (5βA/5βP and 5αA/5βP) for multiple urine samples obtained from two athletes permitted to take testosterone for medical purposes and four athletes whose drug-taking behavior was unknown.

					$\delta^{13}C$[c]			Difference,[c] ‰		Ratios[c]	
Athlete	Day[a]	[T],[b] μg/L	[E], μg/L	T/E	5βA	5αA	5βP	5βP − 5βA	5βP − 5αA	5βA/5βP	5αA/5βP
1	0	2.3	~0.1	20[d]	NA[e]	NA[e]	NA[e]				—
	1	40	1.0	40	−30.42	−31.96	−25.67	4.8	6.3	1.19	1.25
	4	186	2.6	72	−31.10	−31.76	−24.57	6.5	7.2	1.27	1.29
2	0	27	0.9	29	−31.43	−34.57	−26.14	5.3	8.4	1.20	1.32
	28	100	5.1	20	−28.56	−31.04	−24.00	4.6	7.0	1.19	1.29
	63	56	5.7	9.8	−28.66	−30.76	−22.97	5.7	7.8	1.25	1.34
3	0	343	4.3	80	−28.76	−31.25	−23.06	5.7	8.2	1.25	1.36
	56	690	8.9	77	−28.64	−31.10	−22.60	6.0	8.5	1.27	1.38
	70	598	13.7	44	−29.54	−32.30	−22.30	7.2	10.0	1.32	1.45
	156	820	14.6	56	−29.84	−31.33	−21.67	8.2	9.7	1.38	1.45
4	0	312	29.9	10.4							
	273	128	0.5	10.9	−25.32	−25.76	−24.54	0.8	1.2	1.03	1.05
	357	197	20.4	9.7	−24.96	−26.07	−23.43	1.5	2.6	1.07	1.11
	393	166	24.6	6.8							
	428	394	41.3	9.5	−23.55	−26.14	−22.47	1.1	3.7	1.05	1.16
	461	81	16.0	5.0							
	592	383	43.0	8.9							
	656	211	24.8	8.5							
5	0	108	8.6	12.6							
	64	33	3.5	9.4	−24.82	−25.47	−23.49	1.3	2.0	1.06	1.08
	345	130	15.0	8.6	−24.69	−25.52	−23.27	1.4	2.3	1.06	1.10
	412	36	3.7	9.8	−26.91	−24.50	−24.85	2.1	−0.4	1.08	0.99
	436	35	3.8	9.1							
	798	59	11.4	5.1	−26.66	−27.29	−24.77	1.9	2.5	1.08	1.10
6	0	49	6.3	7.8							
	38	46	6.5	7.1	−24.62	−26.04	−23.36	1.3	2.7	1.05	1.11
	73	11	1.3	8.4							
	103	92	10.3	9.0	−25.12	−25.04	−23.52	1.6	1.5	1.07	1.06
Means, athletes 1–3					−29.84	−31.83	−23.97	5.9	7.9	1.25	1.33
Means, athletes 4–6					−25.08	−25.74	−23.67	1.4	2.1	1.06	1.09
z-Scores, athletes 1–3					4.5	8.0		6.5	9.1	6.6	8.9
z-Scores, athletes 4–6					0.7	0.9		−0.02	−0.02	0.30	0.40

[a] Day of urine collection relative to the first sample (day = 0).
[b] [T] and [E], testosterone and epitestosterone.
[c] Cells >3 SD, shaded; cells <3 SD, solid outline.
[d] Value is an approximation because of large error in measurement of epitestosterone.
[e] Unable to measure because of low response (<0.3 V).

units). For subject 3, the urine testosterone concentrations (mean, 613 μg/L; z-score = 2.6) and T/E ratios (mean = 64) were very high. In addition, like athletes 1 and 2, athlete 3 was characterized by low 5βA and 5αA values, and large differences and ratios outside the ± 3 SD range.

In contrast to athletes 1–3, for athletes 4–6, the values for 5βA, 5αA, and 5βP and their differences and ratios were similar to the averages for the control group, and none of the values were remarkable except for one 5βP − 5αA value of −0.4‰ and one 5αA/5βP ratio of 0.99, which were slightly lower than the mean − 3 SD. For subjects 4–6, 5βP − 5βA and 5βP − 5αA were less than 2.1‰ and 3.7‰, respectively, i.e., within ± 3 SD of the control group values. Similarly, all but one of the ratios to 5βP were inside the ± 3 SD range. In addition, the urine testosterone concentrations for subjects 5 and 6 were within + 1.5 SD of the log mean for 11 938 athletes. Thus, compared with the control group, athletes 5 and 6 were characterized by elevated T/E ratios; whereas the values for 5βA, 5αA, and 5βP, their differences, and their ratios for athletes 5 and 6 were, with two minor exceptions, within ± 3 SD of the means of the controls. Athlete 4 had a relatively high mean urine testosterone (234 μg/L; z-score = 1.9).

The use of testosterone or testosterone precursors is indicated by low 5αA and 5βA values, large 5βP − 5αA

and 5βP − 5βA differences, and large 5αA/5βP and 5βA/5βP ratios. Of these six variables (two $\delta^{13}C$ values, two differences, and two ratios), for athletes 1–3, the ratio 5βA/5βP (z-score = 8.9) and the difference 5βP − 5βA (z-score = 9.1) were the most sensitive indicators of testosterone administration as judged by z-scores. The value for 5αA was also a good indicator (z-score = 8.0). For differences and ratios that utilized 5βA (5βP − 5βA and 5βA/5βP), the z-scores were 6.5 and 4.5, respectively. The least discriminating variable was 5βA (z-score = 4.5). In contrast, for athletes 4–6 in Table 5, the z-scores were all low (range, −0.02 to 0.9).

Discussion

CHARACTERISTICS OF THE GC-C-IRMS ASSAY

GC-C-IRMS measurement of urinary steroids has become a potent analytical method in doping control, although scrupulous attention to detail is necessary. Successful implementation of the method requires strict adherence to QC, system suitability, and batch acceptance criteria such as those described herein. In the present study, after the tolerance ranges for the system and batches were established, the system suitability test never failed and the batch acceptance criteria were always met over the 14 months of the study.

In the instrument precision study, the mean SDs were less than ± 0.33‰; thus, when the variability attributable to extraction and derivatization was eliminated, all of the SDs were low. In the within-assay study, the SDs were also low: ± 0.27‰, ± 0.38‰, and ± 0.28‰ for 5βA, 5αA, and 5βP, respectively. The between-assay SDs ranged from ± 0.40‰ to ± 0.52‰, which is slightly higher than the within-assay values, but they indicate the excellent repeatability and reproducibility of the entire assay.

We did not find comparable precision studies for urinary steroids in the literature; however, Shackleton et al. *(3)* provided evidence that $\delta^{13}C$ values for diols are reproducible based on duplicate analysis of 13 samples from one subject. We used the data in Table 1 from Shackleton et al. *(5)* to calculate the SD of duplicates and found values of ± 0.36‰, ± 0.15‰, and ± 0.28‰ for 5βA, 5αA, and 5βP, respectively. These data show that the SD for duplicates in another laboratory was similar to the SD that we obtained for duplicates in our batch acceptance studies. The concentrations of 5β-androstane-3α,17β-diol and 5α-androstane-3α,17β-diol in the control urines were 23–430 μg/L; thus, given the 85% recovery of steroids from the 10-mL sample volume used in the analysis, the concentrations of 5β-androstane-3α,17β-diol and 5α-androstane-3α,17β-diol in the 25 μL of the injection solution were 8–146 mg/L, and the samples were analyzed in the linear range of the system (Fig. 1).

HEALTHY CONTROL MALE SUBJECTS

The $\delta^{13}C$ values in Table 3 represent the largest group of healthy males living in the US measured to date. The ranges for the T/E ratios (0.1–3.6) and urine testosterone concentrations (2–116 μg/L) are consistent with the fact that no student declared taking testosterone or a steroid supplement. In addition, none of the students declared any endocrine disorders. Of the 74 urines, we were able to measure 5βA, 5αA, and 5βP values for all but 1. Sixty-eight of the samples provided satisfactory data on the first extraction of 10 mL of urine. For the other six, three provided an adequate signal on repeat analysis. Two samples required a 20-mL sample volume to obtain an adequate signal. In one sample with low diol concentrations (<25 μg/L), the voltage criteria were not met even with a 20-mL sample. Thus, our signal criteria were met on the first analysis with 10 mL of urine in 94% of the 74 cases, and all but 1 of the remaining samples provided satisfactory data on repeat analysis with 10 or 20 mL of urine. One person with one instrument can perform approximately three assays per week with a batch size of 20 samples plus 5 controls. The software to process the samples is somewhat cumbersome; thus, ~2 days per week are needed for data processing.

The mean 5αA and 5βA values for our control group were −26.35‰ and −25.69‰, respectively. The mean δ values − 3 SD for 5βA and 5αA were approximately −28.4‰, which is similar to the δ values reported for synthetic testosterone *(6, 7)*; however, this comparison should not be made. This is because the diols measured herein were acetylated, which makes the δ value more negative by approximately two units, whereas the synthetic testosterone *(6, 7)* was underivatized.

There are no comparable control group values in the literature; however, the baseline values in Fig. 6 of Shackleton et al. *(3)*, which was based on analysis of 20 samples from individuals with mixed nationalities, are very similar to ours. Their lowest value for a diol was −28.2‰, whereas our lowest diol value was −27.89‰. Ueki and Okano *(7)* also measured 5αA and 5βA; however, their data are not directly comparable to ours because they used a correction factor to convert all measured $\delta^{13}C$ values of the acetylated compounds to give values for underivatized steroids, which we could not estimate. The correction factor would be the same for 5αA and 5βA; thus, their difference of 2.6‰ between the means of 5αA (−16.4‰) and 5βA (−19.0‰) for 10 Japanese male samples can be compared to our difference of 0.66‰ (Table 3). In addition, the value for 5αA was lower than 5βA in the study by Ueki and Okano *(7)*, which is the opposite of what we found. We can also compare the range of values in our control subjects to the range reported by Ueki and Okano *(7)* in their Table 2 for 20 healthy Japanese males. Our ranges for 5αA and 5βA were 2.9‰ and 3.9 ‰ (Table 4), whereas they found ranges of 10.8‰ and 7.2‰, respectively. The striking difference between the ranges found in the two laboratories could reflect differences in method, calibration, ethnicity, or diet. The explanation that Japanese subjects have different steroid biochemistry and metabolism was deemed unlikely because equally large ranges of 11.0‰ (5βA) and 14.1‰ (5αA) were

reported for urine samples from >350 Olympic athletes, and Olympic athletes are multiracial. In addition, Shackleton et al. (3) found similar values for Chinese and other nationalities, and we found no difference among four ethnic groups in our control group. That dietary factors could explain the differences was also considered, but diet is an unlikely explanation for the large differences in Olympic athletes because it is likely to take several weeks on a local diet before a measurable change in urinary steroid $\delta^{13}C$ develops. Therefore, it is likely that our analytical method differs from that of Ueki and Okano (7).

The diversity of the control group was an advantage in that four major ethnic groups were present; however, it was a disadvantage because the n for each ethnic group was relatively small and therefore the statistical power of the GLM procedure was relatively low. Thus, we could not attribute any differences to ethnicity. Similarly, Shackleton et al. (5), who compared $\delta^{13}C$ values for 20 individuals from 12 nationalities, did not attribute any of the differences to ethnicity or diet.

In the present study, the mean $\delta^{13}C$ values for 5αA (−26.35‰) and 5βA (−25.69‰) in the control group were different ($P < 0.001$), whereas in our previous study with only 10 subjects, we did not observe such a difference (15). In addition, it appears from the data in Table 5 of the present study and from Table 1 in our previous study (15) that testosterone administration may decrease 5αA more than it decreases 5βA. This difference occurs despite the fact that the percentage of conversion of a tracer dose of [^{14}C]testosterone to 5β-androstane-3α,17β-diol (3.2%) is greater than the percentage of conversion to 5α-androstane-3α,17β-diol (1.2%) (18). Urinary 5α-androstane-3α,17β-diol is known to arise from both hepatic and peripheral metabolism, whereas urinary 5β-androstane-3α,17β-diol is considered to arise only from hepatic metabolism (19). After pharmaceutical doses of testosterone, 5αA may decrease more than 5βA because peripheral paths to 5α-androstane-3α,17β-diol are favored. However, there is also a path from dehydroepiandrosterone sulfate to 5β-androstane-3α,17β-diol that does not pass through testosterone (20). Utilization of this path could explain why 5βA is higher than 5αA. Finally, the observed differences may be an analytical artifact. Similarly, the higher mean $\delta^{13}C$ value for 5βP (−24.26‰) might be related to metabolism or artifacts.

ATHLETES WITH ELEVATED T/E RATIOS

To clarify the status of an athlete with an elevated T/E ratio, the International Olympic Committee recommends that additional samples be obtained (9). The relevant sport authority typically charts the course of the urine T/E values over time and interprets the data pattern to decide whether to penalize the athlete. Unless there is a record of three or more past samples, this involves collecting additional samples. There are no guidelines stating how many samples are needed, how often they should be collected, and for how long, and there are no published criteria for determining whether the elevated T/E ratio is natural or is attributable to taking an exogenous substance. The data in Tables 1–3 and 5 support the argument that by measuring $\delta^{13}C$, the decision to penalize the athlete can be made on a single sample. The six athletes described in Table 5 were undergoing such testing, although at the time of the analysis the laboratory was not aware that subjects 1 and 2 were permitted testosterone users. No additional information was available on the status of athletes 3–6; however, inspection of the data for subject 3 indicates that it is reasonable to classify him as a testosterone or testosterone precursor user.

The six subjects in Table 5 with increased T/E ratios may be characterized by the concentration of testosterone in their urines and by their 5βA and 5αA values and the associated differences and ratios to 5βP. A classification based on $\delta^{13}C$ values, differences, and ratios revealed that athletes 1–3 were outside the control range ± 3 SD except for 5βP. For athletes 1 and 2, this fits with our understanding of the basis of the carbon isotope ratio method, specifically, that after testosterone administration only the $\delta^{13}C$ values of testosterone and testosterone metabolites will decrease and that those of other urinary steroids will not. Because 5β-pregnane-3α,20α-diol is not a metabolite of testosterone, its $\delta^{13}C$ values are not expected to change with testosterone administration (3, 4, 15).

Applying the means ± 3 SD criteria to the $\delta^{13}C$ data for athlete 3 requires classifying him as a testosterone or testosterone precursor user. This possibility is further supported by the very high urine T/E ratios and testosterone concentrations. The *z*-scores for his urine testosterone concentrations range between 2.2 and 2.9, which is a further indication of testosterone or testosterone precursor administration. Once the GC-C-IRMS method is validated to the point of full acceptance by the sport and legal community, we expect that athlete 3 could be classified as a user on one urine showing the pattern of a high T/E ratio, high testosterone concentration, and $\delta^{13}C$ values less than − 3 SD.

Athletes with naturally elevated urine T/E ratios would be expected to have persistently elevated T/E ratios, unremarkable urine testosterone concentrations, and values for 5βA and 5αA that are within ± 3 SD of the mean of the control group. The data for athletes 5 and 6 fit this pattern. Athlete 4 also fits, but some of his urine testosterone concentrations were relatively high. The *z*-score for the values 383 and 394 μg/L was ~2.3, and all but one of the others were >1.3. Until additional data are available, athlete 4 has been classified as having a naturally elevated T/E ratio, although we cannot completely exclude the possibility that athlete 4 is a testosterone or testosterone precursor user with normal $\delta^{13}C$ values.

The data on mean *z*-scores in Table 5 reveal that both the differences and ratios are better indices of testosterone use than the absolute $\delta^{13}C$ values, 5βA and 5αA. This is

USADA 0818

not unexpected given that 5βP correlates with both 5βA and 5αA. Furthermore, the difference 5βP − 5βA and the ratio 5βA/5βP are more robust indicators of testosterone use than the corresponding differences 5βP − 5αA and the ratio 5αA/5βP. This follows from the finding (Tables 3 and 5) that the $\delta^{13}C$ values for 5αA were lower than the values for 5βA.

In conclusion, a fundamental issue in doping control is whether GC-C-IRMS techniques will resolve ambiguities in the interpretation of the T/E test. The methods presented herein provide compelling evidence that the test has excellent precision and that when it is coupled with strict system suitability and batch acceptance criteria, it can be used in a routine fashion to obtain valid $\delta^{13}C$ data. By comparing the $\delta^{13}C$ values of a control group to data obtained from athletes, it is possible to make informed decisions regarding the origin of urinary 5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol, and 5β-pregnane-3α,20α-diol.

We are grateful for financial support from the National Collegiate Athletic Association, the National Football League, and the United States Olympic Committee. We thank K. Schramm for technical assistance.

References

1. Coppen JJW. Steroids: from plants to pills—the changing picture. Trop Sci 1979;21:125–41.
2. Smith BN, Epstein S. Two categories of $^{13}C/^{12}C$ ratios for higher plants. Plant Physiol 1971;47:380–4.
3. Shackleton CHL, Phillips A, Chang T, Li Y. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids 1997;62:379–87.
4. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG. Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. J Mass Spectrom 1996;31:169–76.
5. Shackleton CH, Roitman E, Phillips A, Chang T. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: potential use in gas chromatography isotope ratio mass spectrometry. Steroids 1997;62:665–73.
6. Horning S, Geyer H, Flenker U, Schänzer W. Detection of exogenous steroids by $^{13}C/^{12}C$ analysis. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U, eds. Recent advances in doping analysis, Vol. 5. Köln: Sport und Buch Strauss, 1997:135–48.
7. Ueki M, Okano M. Analysis of exogenous dehydroepiandrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 1999; 13:2237–43.
8. Donike M, Bärwald KR, Klostermann K, Schänzer W, Zimmermann J. Nachweis von exogenem Testosteron. In: Heck H, Hollmann W, Liesen H, Rost R, eds. Sport: Leistung und Gesundheit. (Kongressbd. Deutsche. Sportarztekongress) Köln: Deutsche Ärzte-Verlag, 1983:293–8.
9. International Olympic Committee. List of doping classes and methods. Lausanne, Switzerland: IOC, 1992.
10. Falk O, Palonek E, Björkhem I. Effect of ethanol on the ratio between testosterone and epitestosterone in urine. Clin Chem 1988;34:1462–4.
11. Oftebro H. Evaluating an abnormal urinary steroid profile. Lancet 1992;339:941–2.
12. Catlin DH, Hatton CK, Starcevic S. Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athletes' urine. Clin Chem 1997;43:1280–8.
13. Dehennin L, Matsumoto AM. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. J Steroid Biochem Mol Biol 1993;44: 179–89.
14. Becchi M, Aguilera R, Farizon Y, Flament M-M, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. Rapid Commun Mass Spectrom 1994;8: 304–8.
15. Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, Swerdloff RS, et al. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. J Chromatogr B 1999;727:95–105.
16. Catlin DH, Kammerer RC, Hatton CK, Sekera MH, Merdink JM. Analytical chemistry at the Games of the XXIIIrd Olympiad in Los Angeles, 1984. Clin Chem 1987;33:319–27.
17. Koch DD, Peters TP. Selection and validation of statistical methods. In: Burtis CA, Ashwood ER, eds. Fundamentals of clinical chemistry, 2nd ed. Philadelphia: WB Saunders, 1996:170–81.
18. Baulieu EE, Robel P. Catabolism of testosterone and androstenedione. In: Eik-Nes KB, ed. The androgens of the testis. New York: Marcel Dekker, 1970:49–71.
19. Mauvais-Jarvis P, Floch H, Jung I, Robel P, Baulieu EE. Studies on testosterone metabolism. VI. Precursors of urinary androstanediols. Steroids 1968;11:207–24.
20. Mauvais-Jarvis P, Baulieu EE. Studies on testosterone metabolism. IV. Urinary 5α- and 5β-androstanediols and testosterone glucuronide from testosterone and dehydroisoandrosterone sulfate in normal people and hirsute women. J Clin Endocrinol Metab 1965;25:1167–78.

ELSEVIER

Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols

Cedric H.L. Shackleton,* Andy Phillips,† Tony Chang,* and Ye Li‡

**Children's Hospital Oakland Research Institute, Oakland, California, USA; †Micromass UK Limited, Manchester, England, UK; and, ‡National Research Institute of Sports Medicine, Beijing, China*

A gas chromatographic combustion isotope ratio mass spectrometric (GC/C/IRMS) method was used for studying the incorporation of exogenous testosterone enanthate into excreted urinary 5α- and 5β-androstane-3α,17β-diols. A multistep but straightforward work-up procedure produced a simple GC chromatogram of urinary steroid acetates composed principally of two androstanediols and pregnanediol. It is anticipated that such a method may form the basis of a doping control test for testosterone that could be used as a primary method during major sporting events or alternatively as a verification technique. Urine samples from five individuals were collected before and after administration of testosterone enanthate (250 mg). The $\delta^{13}C$ °/00 value of androstanediols was around −26 to −28 during the baseline period and decreased to about −29 to −30 in the days following synthetic testosterone administration. One of the other major steroids in the chromatogram, pregnanediol, was utilized as the "internal standard," because its $\delta^{13}C$°/00 values did not markedly change following testosterone administration, remaining at −25 to −27. In all subjects studied, the $\delta^{13}C$°/00 values for androstanediols were reduced sufficiently over 8 days to confirm administration of synthetic testosterone. Although steroids isolated from urine of normal individuals from 12 different countries gave values between −24 and −28, this seemed not to be related to nationality or region. The most likely variable is the proportion of plants with low and high carbon 13 content in the diet. This variable is likely to be more affected by individual food preferences than broad ethnic food divisions. In this paper, we propose a ratio of $\delta^{13}C$°/00 for androstanediols to pregnanediol as a useful discriminant of testosterone misuse, a value above 1.1:1.0 being indicative of such misuse. The work-up procedure was designed for batch analysis and to use only simple techniques, rather than employ further instrumentation, such as high-performance liquid chromatography (HPLC), in purifying steroids for GC/C/IRMS. (Steroids **62**:379–387, 1997) *© 1997 by Elsevier Science Inc.*

Keywords: isotope ratio mass spectrometry (IRMS); doping control; testosterone

Introduction

The increasing need in sport for proving testosterone misuse requires new methodologies. For many years, a testosterone/epitestosterone (T/E) excretion ratio determined by gas chromatography–mass spectroscopy (GC–MS) of greater than 6:1 has been used as the hallmark for confirmation of drug administration,[1] but this method is fallible. For one thing, occasional drug-free individuals give a ratio >6, and a high ratio can also be adjusted downward by simultaneous administration of epitestosterone. We have found that in eight Chinese subjects given 250 mg testosterone enanthate, only three gave T/E values >6 on more than 1 day, demonstrating a high rate of false negatives, at least in this racial group.

In 1990, Southan and co-workers[2] used isotope ratio mass spectrometry (IRMS) to show that synthetic testosterone had a different ^{13}C content than endogenous hormone. This is a reflection of the origin of the materials, because all testosterone, both endogenous and synthetic, is ultimately of plant origin. Gonadal testosterone is made from precursor molecules derived from a wide variety of vegetable mate-

Address reprint requests to Cedric H.L. Shackleton, Children's Hospital Oakland Research Institute, 747 52nd Street, Oakland, CA 94609, USA.
Received July 25, 1996; accepted November 4, 1996.

Steroids 62:379–387, 1997
© 1997 by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0039-128X/97/$17.00
PII S0039-128X(96)00253-X

rials eaten by humans or by the animals humans eat. Individual plants discriminate to different degrees against $^{13}CO_2$, and particular species are known to have high or low levels of ^{13}C in their biomolecules.[3] In human bodies, the ^{13}C content, therefore, reflects an average of the ^{13}C content of all the plant material eaten by humans and our animal protein providers. Synthetic testosterone, in contrast, is generally made from a single plant species, mostly soy, so the ^{13}C content has a defined value reflecting the relatively low ^{13}C content of this plant. Thus, a significant difference in ^{13}C content between gonadal and soy testosterone could provide the basis for developing a definitive test for hormone misuse. Differences in carbon isotope ratios referred to by the symbol δ, defined as the difference in isotope ratio between the sample and an international carbonate standard "PDB." Although this is the accepted standard, for common usage a calibrated international standard of CO_2 is used. The values reported for δ carbon isotope ratios are

$$\delta^{13}C^{0}/_{00} = \left(\frac{\text{Ratio sample} - \text{Ratio PDB}}{\text{Ratio PDP}}\right) \times 1000$$

Becchi and coinvestigators have published pioneering studies on development of a method employing IRMS for determination of carbon isotope ratio of testosterone extracted from urine.[4,5] They demonstrated that, providing sufficient urine was available, the endogenous or exogenous origin of testosterone could be readily determined. A major remaining problem demonstrated by these studies relates to sensitivity of the analysis, because the quantity of urine collected from athletes is relatively small (about 75 mL), and this is divided into two, a primary (A) and a secondary sample (B). On each sample, nonsteroidal drug metabolites must be analyzed, as well as anabolic steroid screening and measurement of the T/E ratio.

We have attempted to improve the methodology to allow more sensitive analysis. It was decided to forgo any attempt to analyze testosterone itself and to concentrate on analysis of its metabolites 5α-androstane-3α,17β-diol (5αAD) and 5β-androstane-3α,17β-diol (5βAD). Our overall objective was to easily produce a single sample for analysis containing a few defined steroids to include the androstanediols and steroids we call "endogenous reference compounds" (ERCs). An ERC, in this instance, is a steroid whose carbon isotope ratio could not be altered through administration of exogenous testosterone. Aguilera, Becchi, and co-workers in their most recent publication use cholesterol and 5-androstene-3β,17β-diol as ERC.[5] To achieve our overall objective, we developed simple methodology adaptable to batch analysis, which required no liquid chromatographic (HPLC) instrumentation. Using this methodology, we determined the $\delta^{13}C^{0}/00$ of the androstanediols present in urine following administration of testosterone enanthate to five volunteers. This communication presents the results of this study.

Experimental

Materials

Testosterone enanthate, Testoviron depot® was obtained from Schering, Japan. Reference steroids were obtained from Sigma (St. Louis, Missouri, USA), which was also the supplier of Girard reagent T (carboxymethyl, trimethyl ammonium chloride hydrazide) and sodium bismuthate. Sephadex LH 20 was a product of Pharmacia AB and Sep-pak® cartridges, a product of Waters Corp. (Milford, Massachusetts, USA). β-glucuronidase/aryl sulfatase was obtained from Sigma (Type H1) and Boehringer Mannheim (Mannheim, Germany). Solvents were of analytical grade.

Individuals studied

Eight Chinese male subjects aged 19–22 were studied, although GC/C/IRMS analysis was only conducted on five. Permission for undertaking these experiments was obtained from the Chinese National Research Institute of Sports Medicine, and consent was obtained from the participants. Spot morning urine samples were collected for 2 days prior to an intramuscular injection of 250 mg testosterone enanthate. Two urine samples (0–8 h and 8–24 h) were collected for the first 4 days after administration, although only aliquots of the early morning sample were subject to analysis. Morning spot urine samples were collected on the 5–9th days after administration and on days 11, 13, and 15.

Determination of testosterone/epitestosterone ratio (T/E)

Urinary testosterone and epitestosterone were quantified in all the samples using an adaptation of the method of Donike et al.[6] These measurements were carried out by one of us (YL) in China at the National Institute of Sports Medicine. T/E ratios were then determined.

Preparation of steroid extract for GC/C/IRMS

A flowsheet summarizing the methodology is shown in Figure 1. Urine (typically 25 mL) was extracted by Sep-pak® cartridge according to the method of Shackleton and Whitney.[7] Once dried, the extract was dissolved in 3 mL 0.1 M acetate buffer pH 5 and *Helix pomatia*-derived β-glucuronidase/sulfatase (12 mg Sigma

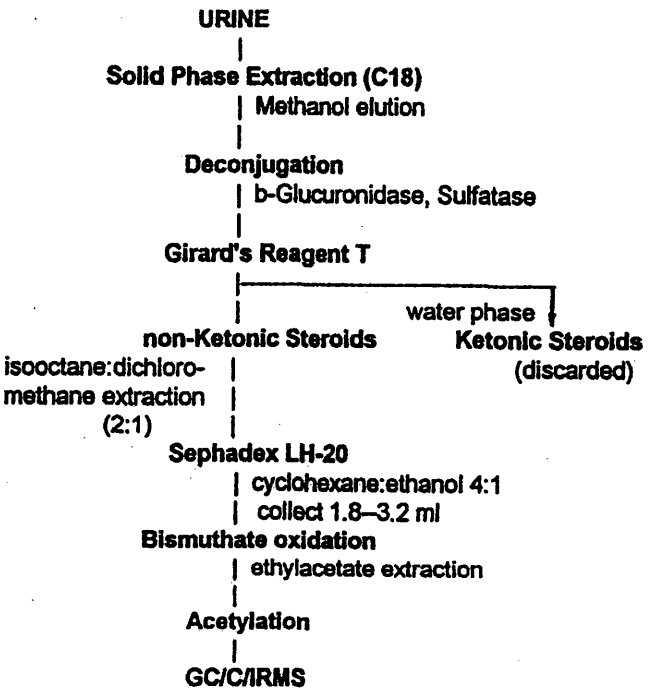


Figure 1 Flow sheet of the urinary extraction method.

USADA 0821

type H1 powder, 100 μL Boehringer liquid enzyme) was added. Hydrolysis was allowed to proceed for 3 h at 55°C. A Girard separation was carried out to separate carbonyl-containing steroids (ketonic) from noncarbonyl-containing (nonketonic) steroids.[8,9] To the hydrolyzed urine mixture, 2 mL glacial acetic acid and 100 mg Girard reagent T was added. The solutions were placed in an oven at 100°C for 30 min. The nonketonic steroids were extracted by 2 × 5 ml isooctane: dichloromethane 2:1 (v/v), and the solvent was dried. Small columns of 0.5 Sephadex LH-20 were prepared in Pasteur pipettes, the Sephadex being allowed to swell in the cyclohexane: ethanol (4:1) solvent system before preparation.[10] The steroid extract dissolved in 100 μL of the same solvent mixture was added to these columns. Solvent eluting between 1.8 and 3.2 mL was collected and dried. Acetic acid (0.1 mL), water (0.1 mL), and 5 mg sodium bismuthate were added.[9] Oxidation was allowed to proceed for 2 h, and after neutralization (0.5 mL of 0.5 M acetate buffer), the mixture was extracted with 4 mL ethyl acetate. After drying, steroid acetates were prepared overnight with 50 μL acetic anhydride and 50 μL pyridine. The acetates were analyzed by GC/C/IRMS.

Gas chromatography combustion isotope mass spectrometry (GC/C/IRMS)

A schematic representation of the GC/C/IRMS instrumentation is illustrated in Figure 2. The acetylated steroid samples were kept refrigerated until analysis. Cyclohexane (20 μL; 99.9% pure from Sigma Chemicals 27-0625-8) was added to each vial, and one-tenth (2 μL) of each sample was injected splitless onto a J&W 30 m DB17 capillary column housed in a Fisons 8000 series GC. The injector was kept at 220°C. The temperature program was as follows: starting temperature 50°C (1 min), followed by rapid temperature increase (25° min) to 300°C, where it was held for 15 min.

The separated components were heart-split into the combustion furnace filled with copper oxide wires (Elemental Microanalysis Limited, UK) held at 850°C. The combustion gases were passed through a nafion membrane water removal trap, and the remaining CO_2 was analyzed on a Micromass isochrom isotope mass spectrometer. The mass spectrometer consisted of an electron impact source running at 400 μA trap current, the ionized CO_2 gas, was focused by a magnet onto three Faraday collectors. The ions collected were those at masses 44, 45, and 46. The Micromass data system calculated the areas of the beams and subtracted any background; whereupon, calculation of the ^{13}C δ values for the successive peaks were carried out.

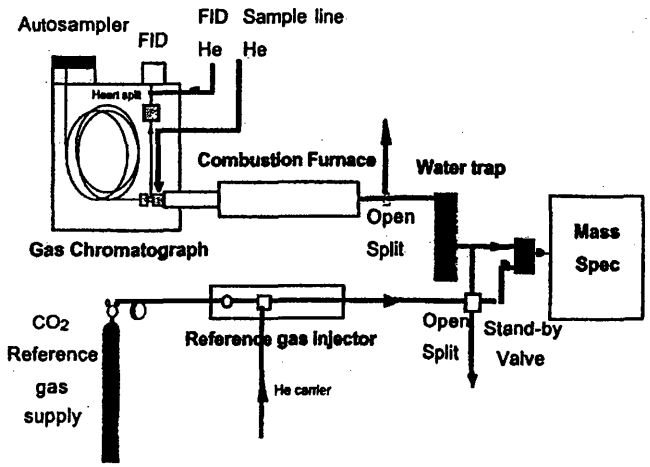


Figure 2 Schematic of the Micromass GC/C/IRMS instrument.

Results and discussion

Evaluation of gas chromatographic columns

The objective of the study was to measure $\delta^{13}C^0/00$ for urinary androstanediols formed as metabolites of testosterone. In our initial studies (Subject 1), we undertook chromatography on DB1 capillary columns but did not get resolution of the two diacetylated epimers (Figure 3A). Later employment of a DB17 column (Figure 3B) allowed separation of the epimers as well as the ERC, pregnanediol diacetate, and pregnanetriol diacetate (Figure 3B).

Confirmation of identity of steroids in extracts

Prior to sending the first samples for GC/C/IRMS analyses in England, the identities of the principal components of the chromatogram were confirmed by GC/MS. This was carried out on a Hewlett-Packard 5970 instrument housing a 15 meter DB1 capillary column. The peaks chosen for GC/C/IRMS analyses had retention times and electron impact mass spectra identical to those of 5α- and 5β-androstanediol diacetate and pregnanediol diacetate. Reference steroids for these compounds were also analyzed on the GC/C/IRMS instrument using both DB1 and DB17 columns, and these gave identical retention times to the urinary steroids. Pregnanetriol could also be analyzed by GC/C/IRMS.

Achievement of work-up procedure objective

The method developed and utilized had the following qualities. First, the Girard separation almost completely removed carbonyl containing steroids from the hydrolyzed extracts, which probably represent 75% of urinary steroids. Exceptions may be the 11-carbonyl containing steroids that probably do not react because of the hindered nature of that functional group. Second, a crude micro Sephadex LH-20 column separation effectively produced a fraction containing steroids with two and three functional groups. Third, sodium bismuthate oxidation was designed into the procedure as a means of removing remaining long-retention time pregnane metabolites, thus allowing shorter periods between injection. Many of the quantitatively more important metabolites are converted into 17-oxygenated C_{19} steroids by the procedure. Fourth, acetylation provided steroids with good gas chromatographic properties that were readily separated. Fifth, despite the complexity of the steroid fraction of urine, the final chromatogram was simple and composed of only a few peaks for which $\delta^{13}C^0/00$ could be determined with accuracy.

$\delta^{13}C^0/00$ value of the synthetic testosterone

Testosterone acetate prepared from the Japanese testosterone enanthate used for injection in these studies gave a $^{13}C^0/00$ value of −30.41, a value close to the lowest value obtained for androstanediol diacetate measurements obtained in the subjects studied following testosterone administration.

We also analyzed five other current products and one synthetic sample made more than 40 years ago. The following results were obtained: testosterone of Chinese manufacture −30.40; U.S. manufacture −30.38; two Czech products,

USADA 0822

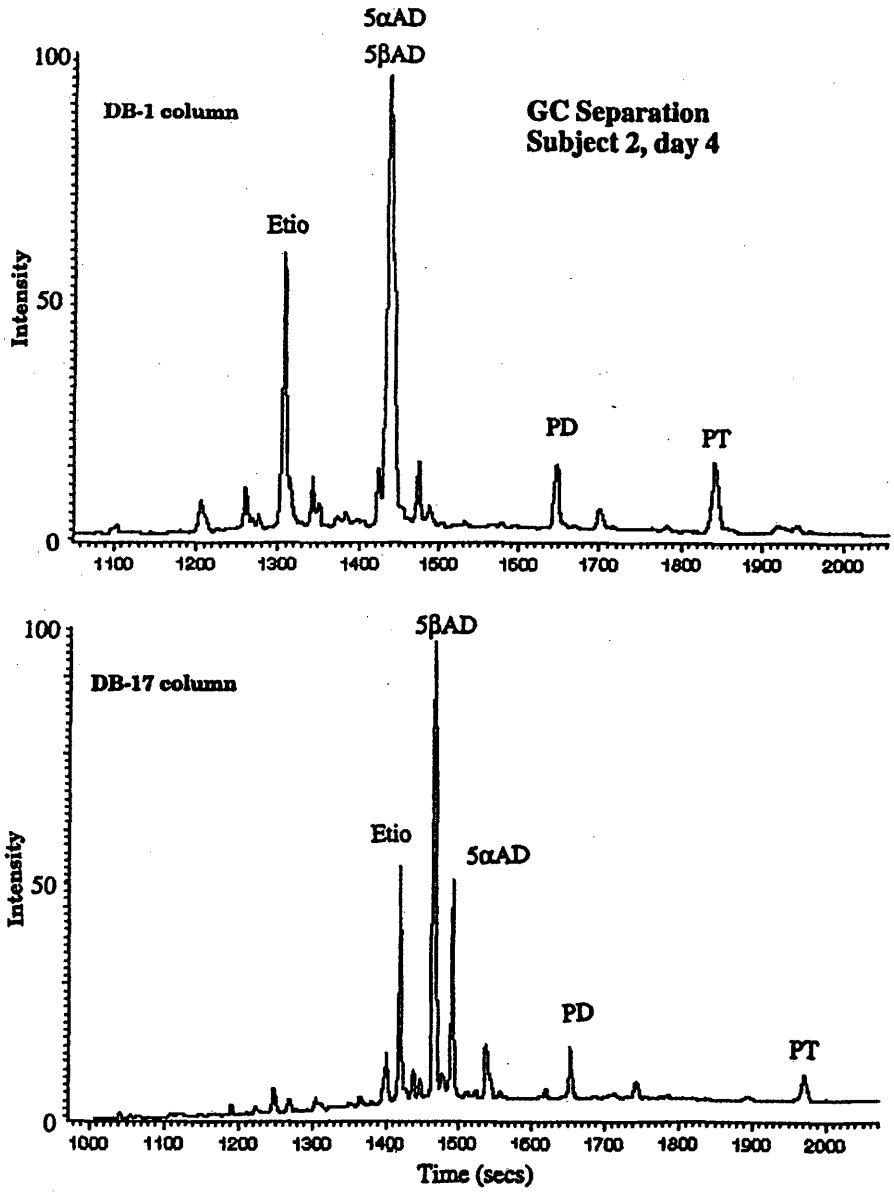


Figure 3 Gas chromatographic separation (on GC/C/IRMS instrument) of acetylated urinary steroid fraction. This sample was initially separated on a DB1 column, which failed to resolve the 5α- and 5β-androstanediols (upper panel). Later employment of a DB17 column permitted individual measurement of the epimers (lower panel). Both columns also resolved etiocholanolone, produced by bismuthate oxidation of pregnanetriol, pregnanediol, and nonoxidized pregnanetriol.

−29.28 and −29.15, respectively; and one Russian −30.22. The U.S. sample of more than 40 years old gave a value of −33.18, probably reflecting the different plant origin of synthetic steroids made long ago. We believe that testosterone is currently made mostly from soy by-products, although originally sterol constituents of the Mexican yam were the primary precursors.

Results for individual subjects

With the exception of Subject 1, a DB17 capillary column was utilized that allowed separate analysis of 5α- and 5-β androstanediols. Graphs illustrating the $\delta^{13}C^{0}/00$ results are shown in Figure 4A–F. In all cases, the $\delta^{13}C^{0}/00$ values for the androstanediols fell significantly following testosterone administration.

Figure 4 illustrates two other features. One is the adequacy of making single measurements of $\delta^{13}C^{0}/00$ for each sample. Panel B and C show results obtained by plotting the average of duplicate measurements (B) and of the *first* measurement of the duplicate pair (C). Essentially these graphs are identical, suggesting duplicate GC/C/IRMS analyses of the same preparative extract are unnecessary. The second feature relates to the trend of slightly increasing δ values for pregnanediol during the study period of Subject 3 (Figure 4D). Because this is not caused by random irreproducibility we proposed that the individual changed his diet substantially to foodstuffs with higher $\delta^{13}C^{0}/00$ values.

USADA 0823

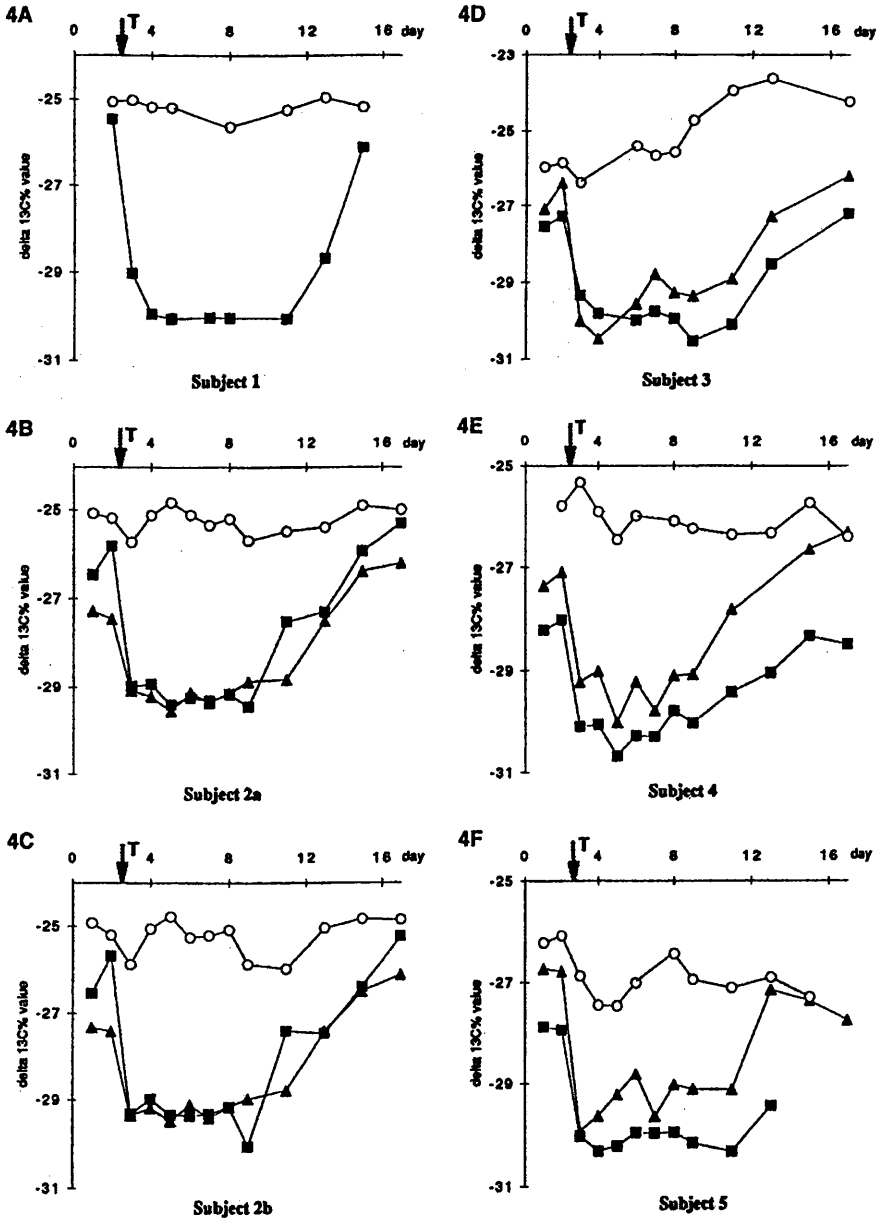


Figure 4 Values of $\delta^{13}C^{0}/00$ for all five subjects throughout the study period. The open circles represent pregnanediol, the closed rectangles 5βAD and closed triangles 5αAD. 4A shows the values obtained for Subject 1 in which only one baseline sample was analyzed, the testosterone being administered on the 2nd day of study. Both androstanediols were measured together on a DB1 column, 4B shows plots for Subject 2 obtained by averaging values for the duplicate runs, and 4C shows equivalent plots through only using the first analysis. 4C, 4D, and 4E are plots of Subjects 3, 4, and 5. For Subjects 2–5 the testosterone was administered after the second baseline sample (day 3 of study).

Designing a method for universal use demands final agreement of numerative values above and below which individuals are considered to have or to have not illicitly used a drug. In our studies with the Chinese subjects, it can be stated that for the five individuals, none had androstanediol $\delta^{13}C^{0}/00$ values less than −28.3 during the control period, and establishing a conservative cut-off value of −29.0 would clearly pick up all drug users for about 7 days after administration. Additionally, we considered that measurement of analyte/ECR ratios could allow numerative values to be established that are to some degree independent of variables introduced through using the methodology in different laboratories with different instrumentation. In Figure 5A, we show the $\delta^{13}C^{0}/00$ ratio of combined androstanediols (average of 5α- and 5β-) to pregnanediol (PD) for the five individuals studied so far. All baseline ratios fell below 1.08, and from these data, we would suggest that a ratio of greater than 1.1 could be used to confirm testosterone administration. When we averaged the data for the five individuals, we found that the AD/PD ratio value of 1.1 was exceeded for 11 days (Figure 5B). Because a combined androstanediol measurement is carried out in the Aguilera et al. studies,[5] comparable data could be prepared from their measurements, although in their case, cholesterol would be the ERC used for ratio determination.

USADA 0824

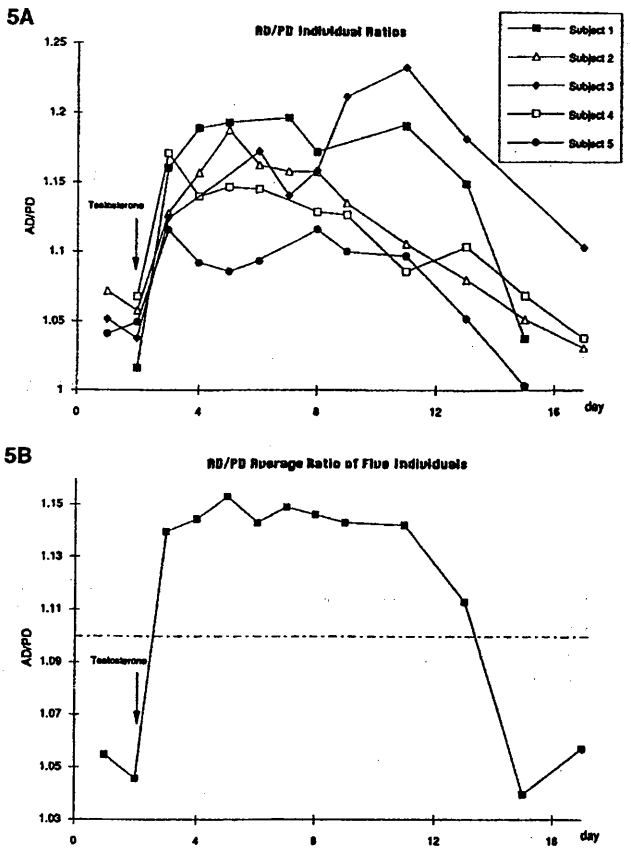


Figure 5 Androstanediol/pregnanediol ratio. A) ratios of $\delta^{13}C^0/00$ for the androstanediols (mean of 5α and β) to pregnanediol for the five subjects; B) average of the ratio for the five subjects for each day; the dotted line represents the proposed discriminant value (1.10) for confirmation of testosterone administration.

Reproducibility of isotope measurements and accuracy

For the five subjects studied, ideally 26 or more isotope measurements were made for each steroid; i.e., 13 duplicate analyses. A few samples were lost or contaminated, and occasionally only single measurements were made on a sample. As a representative analysis, Table 1 gives all of the measurements for Subject 2. Using these data, there are two features of accuracy and reproducibility that can be quantified. One is the variation in δ measurement between duplicate analyses of the same sample and the other the overall variation occurring during analyses of a complete series from one individual. For duplicates, the reproducibility was excellent and did not differ significantly between the five subjects studied. Considering the data shown in Table 1, averages and standard deviations were measured for each duplicate for the two androstanediol and pregnanediol diacetates that give values in the $\delta^{13}C^0/00$ −23 to −30 range. Means were then determined of the standard deviations. For pregnanediol, the mean standard deviation between duplicates was ± 0.21 with a range of ± 0.02 to ± 0.71. Comparable results were obtained for the androstanediols (± 0.11 for 5α-androstanediol and ±0.25 for 5β-androstanediol). These results were considered to represent excellent reproducibility and (as previously noted) suggested that the methodology could be used with *single* determinations by GC/C/IRMS of $\delta^{13}C^0/00$ for each steroid. This was illustrated graphically in Figure 4B and C, which showed both the $\delta^{13}C^0/00$ values for the average of duplicates and for the first analysis. The graphs were essentially identical.

Within a 17-day series of datapoints for duplicate assays for a single individual, the results show considerably less reproducibility—notice the peaks and valleys of the pregnanediol plots in Figure 4. In any physicochemical analyses, it is, of course, possible to have excellent reproducibility but compromised values. For accuracy and reproducibility assessment between a series of different samples, we use the δ measurement of the ERC pregnanediol. The administration of testosterone should in no way change the $\delta^{13}C^0/00$ value for pregnanediol that has to be produced from cholesterol via several intermediates. In a perfect analysis, the measured ^{13}C content of pregnanediol would not be expected to change during the 17-day study period unless, of course, the individual alters his or her diet markedly during the study period. Even with dietary modification, the ^{13}C content of body molecular constituents would be expected to change only slowly.

The highest and lowest duplicate average $\delta^{13}C^0/00$ values for pregnanediol for the five subjects differed by −0.92 to −2.96. As previously mentioned, with the exception of Subject 2, the average duplicate values seemed to rise and fall randomly during the 17-day study period. What

Table 1 $\delta^{13}C^0/_{00}$ values obtained from duplicate GC/C/IRMS analyses for Subject 2 before and following testosterone administration

	Subject 2		
	5βAD	5αAD	PD
Day 1	−26.55	−27.33	−24.93
	−26.15	−27.20	−25.34
	−26.66	−27.28	−25.15
Day 2	−25.68	−27.42	−25.20
	−25.95	−27.49	−25.17
Day 3[a]	−29.33	−29.36	−25.88
	−28.63	−28.81	−25.61
Day 4	−28.99	−29.19	−25.06
	−28.88	−29.24	−25.21
Day 5	−29.34	−29.49	−24.78
	−29.48	−29.61	−24.87
Day 6	−29.37	−29.11	−25.26
	−29.12	−29.16	−24.97
Day 7	−29.33	−29.42	−25.22
	−29.31	−29.28	−25.46
Day 8	−29.16	−29.16	−25.09
	−29.18	−29.14	−25.30
Day 9	−30.06	−28.98	−25.88
	−28.83	−28.78	−25.52
Day 11	−27.41	−28.78	−25.99
	−27.63	−28.88	−24.99
Day 13	−27.47	−27.42	−25.05
	−27.12	−27.59	−25.72
Day 15	−26.39	−26.50	−24.84
	−25.50	−26.28	−24.96
Day 17	−25.24	−26.12	−24.86
	−25.36	−26.32	−25.13

Abbreviations: 5βAD, 5β-androstane-3α,17β-diol; 5αAD, 5α-androstane-3α, 17β-diol and PD, pregnanediol
[a]The first collection following testosterone administration.

USADA 0825

is the source of this irreproducibility? Because we know that the reproducibility of $\delta^{13}C^0/00$ measurements of individual steroids in duplicate GC/C/IRMS runs is excellent, we must assume that the *measured* $\delta^{13}C^0/00$ values for individual chromatographic peaks are accurate. If these are accurate, then any difference in the value from the "true" value for the steroid determined must represent some minor contamination of the steroid peak by components with greater or less $\delta^{13}C^0/00$ values. We have proposed that the trend to increasing values for Subject 2 may be because of dietary changes toward foodstuffs with greater ^{13}C content.

These minor irreproducibilities, which typically give rise to a range of about −1.5 in the δ value for all datapoints during the study period, will not affect the outcome of a drug test, because in no cases do the lowest values breach the discriminant value for the positives.

Testosterone/epitestosterone ratios

Table 2 gives the T/E values for five individuals studied here (Subjects 1–5) and three other subjects whose steroids were not analyzed by the IRMS technique (Subjects 6–8). The data show that the maximal increase in the ratio for each of the individuals was between 12 and 68 times baseline level, although it was noticeable that only 4 of the 8 subjects gave T/E values greater than 6 and 1 of these only modestly exceeded it on one occasion (T/E 6.69). The T/E ratio of the five subjects studied here have been included in Figure 5C as compared to the $\delta^{13}C^0/00$ AD/PD ratio for the same individuals.

$\delta^{13}C^0/00$ steroid values for different nationalities

Figure 6 shows the $\delta^{13}C^0/00$ values for duplicate analyses of the androstanediols and PD from 15 individuals of 11 different nationalities in addition to the Chinese. In some cases, it was possible only to obtain values for the 5β androstanediols because of the low amounts of 5αAD present. The results demonstrate that there are differences among racially and regionally varied individuals with undetermined diet, but we must remember that part of this variation may be attributable to the analytical inaccuracies previously discussed. However, all values were greater than −28.2, which should be compared to the values of less than −29.2

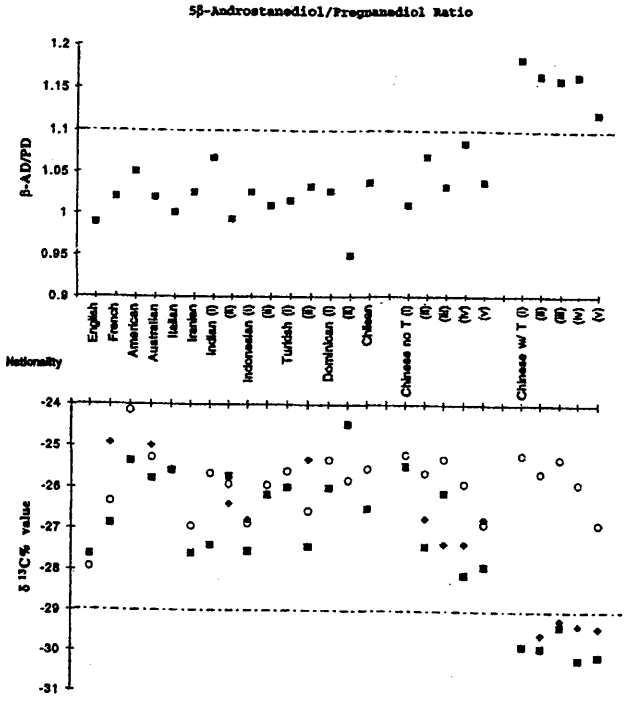


Figure 6 The lower panel shows the $\delta^{13}C^0/00$ values for the androstanediols and pregnanediols for individuals of different nationality, while the upper panel shows the 5βAD/PD ratio. The open circles represent PD, the closed diamonds 5αAD and closed rectangles 5βAD. The baseline values for the Chinese represent the mean of the two measurements, while the Chinese "testosterone" values represent the average of the 7 days after drug administration. Pregnanediol was below detection level in the Chilean urine, so pregnanetriol values were reported instead.

found in the subjects studied following testosterone administration. The mean value for the 5βAD/PD ratio was 1.02 ± 0.03 with a range of 0.95 to 1.07, which compares to the average value for the Chinese baseline samples of 1.05 ± 0.03 (range 1.01 to 1.08). The ratio values for the testosterone administered subjects were all >1.15 in the first days after injection. These data support the previous cut-off ratio value of 1.1:1.0 suggested on the basis of studying the Chinese subjects before and after testosterone administration.

There are notable features with regard to the ^{13}C content

Table 2 Urinary testosterone/epitestosterone ratios for the eight subjects

Study day	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subjec 7	Subject 8
1	0.26	1.28	4.69	0.10	0.12	0.17	0.08	0.11
2	0.33	1.48	5.26	0.11	0.11	0.22	0.06	0.11
3[a]	0.65	8.77	29.3	0.26	0.7	1	1.07	0.42
4	1.95	24.2	36.9	1.77	6.69	4.13	1.36	2.98
5	2.81	45.8	41.8	2.01	5.91	3.53	1.53	7.23
6	2.79	33.2	61.8	1.96	3.71	3.10	1.45	7.48
7	3.03	15.4	54.9	1.91	4.24	3.65	1.68	7.16
8	4.13	36.4	57.7	1.48	5.95	3.08	0.97	4.89
9	5.3	20.3	39.7	1.51	5.78	1.43	1.04	3.81
11	3.2	26.5	33.0	0.58	3.69	0.97	0.40	3.69
13	0.77	2.63	3.42	0.68	3.41	0.20	0.07	1.93
15	0.22	0.99	4.37	0.19	2.27	0.15	0.06	0.21
17	0.26	2.02	4.80	0.13	0.21	0.20	0.08	0.08

[a]Day of testosterone administration.

of steroids in urine from individuals of different countries. One is that there is a considerable spread in values but one that is unrelated to race and "typical regional diets," at least based on the small sample studied. The lowest and highest $\delta^{13}C^0/00$ among this group differed by 4 units (−24 to −28); however, both these sets of values came from Caucasians. The remaining samples from different nationalities and ethnic groups fell within this range, a particularly important finding with respect to the current study, because it suggests that the results obtained following testosterone administration are universally applicable.

Diet of individuals must have the greatest part to play in the establishment of an individual's $\delta^{13}C$ value. Because all food originates as plant life (including animal protein), it was interesting to research what is known of $\delta^{13}C^0/00$ values for dietary plants. Smith and Epstein[3] published the ^{13}C content of 104 plants, but relatively few were commonly used foodstuffs. A few were high $^{13}C/^{12}C$ plants (corn, sugar cane, grain, and sorghum) averaging around $\delta^{13}C^0/00$ −14, but most were categorized as a separate group of low $\delta^{13}C^0/00$ species having values between −23 and −30. These include dietary oil precursors (olive, sunflower, and castor) and various vegetables (wheat, grass, bamboo, peas, squash, radish, beets, and citrus fruit). Because typical worldwide diets of human and domestic animals use mostly low $\delta^{13}C^0/00$ plants, we would anticipate that $\delta^{13}C$ values for excreted steroids would fall in the −23 to −28 range, as found. The $\delta^{13}C^0/00$ value of an individual plant species does not vary significantly dependent on geographic location,[3] and variation only manifests itself in plants grown in urban areas where CO_2 from fossil fuels increases the ^{12}C content slightly. This clearly does not markedly affect agricultural foodstuffs. For the purposes of this assay technique, it is fortuitous that synthetic testosterone is obviously derived from a very low $\delta^{13}C^0/00$ plant species, otherwise no distinction would be possible between endogenous and exogenous compound.

Practicality of methodology for use in doping control

We compared our $\delta^{13}C^0/00$ values for androstanediol with those of Aguilera et al., who used similar methodologies but different instrumentation.[5] They studied individuals of different nationality and ethnicity than ours. For the purposes of this comparison, we averaged our values for 5α- and 5β-androstanediol, because their values were based on measurement of the combination. For baseline samples, we obtained an averaged $\delta^{13}C^0/00$ of −26.87, which agreed excellently with Aguilera's[5] value of −26.52. For androstanediols measured during testosterone administration, they obtained an average value of −32.44; whereas, our value (the mean of duplicates of the lowest values for each individual) was −30.21. Although our minimum value is not as low as that of Aguilera, it was still well below the baseline level, so it could be used for proving testosterone abuse. The difference in results between the laboratories could be attributed to their use of a testosterone with lower $\delta^{13}C^0/00$ (its origin was not reported) or unequal calibration of the different instruments used.

Our methodology is multistep, albeit built around low-technology procedures. Within the technique, there is only one chromatographic separation, and that was designed to produce a broad fraction rather than the isolation of individual steroids. The key to the method is separate isolation of nonketonic steroids, a procedure that in one extraction step removes 75% of unwanted steroids from urine. A second classical procedure, the oxidative removal of side chains from 17-hydroxypregnane steroids removes many of the remaining complex steroids from the mixture, resulting in a cleaner chromatogram, although in the future, we may simplify the method by discontinuing this procedure if satisfactory results can be obtained. Typically, without using any automation in wet chemistry procedures, a batch of samples requires 1 day of technician time to extract and derivatize. The mass spectrometry was automated, and latterly 20 minutes were required for each run. The maximum number of samples that could be analyzed per 24 h was, therefore, 72.

The length of time that the 250-mg testosterone enanthate dose (equivalent to 180-mg free testosterone) clearly manifested itself in decreased $\delta^{13}C^0/00$ value was 8–10 days. This compares well with other parameters studied on this cohort. For example, plasma testosterone was measured, and from being grossly elevated in the first few days after administration, it returned to normal by the 8th day, although the steroid itself may well be largely of exogenous origin on this occasion.[11] In addition, we report the T/E ratios that are particularly interesting, because essentially only three out of the eight individuals achieve values >6 on more than 1 day, showing a high rate of false negatives. This may not be a universal finding and may well be related to the race (Asian) of the studied individuals. However, it once again emphasizes the importance of developing alternate methodology for proving testosterone misuse. The elevated T/E ratios (although most are <6) fall significantly by the 11th day of the study in all subjects.

We believe that our method based on androstanediol measurement has the needed sensitivity for use in doping control. Although we analyzed all samples in duplicate and plotted the graphs from their averages, individual analyses were equally informative (Figure 4B, C). We would have no hesitation in confirming testosterone administration based on a single measurement. We have been able to get up to five analyses of a single sample using splitless gas chromatographic injection. This represents an equivalent of 5 mL urine for each analysis, which meets sample availability in doping control. Use of a solid injection device for GC, such as glass minivials in a carousel,[12] could improve this sensitivity greatly, because the total sample could be volatized and passed into the GC/IRMS instrument. Testing of a urine volume of 2 mL by our procedure and the Micromass IRMS instrument would be realistic. Our method has two obvious advantages over that of Aguilera et al.[5] One is that the two androstanediols are determined separately, so even if one peak was too small or showed evidence of contamination, the second peak could give the required value. A second advantage is that all analytes are measured in the same chromatogram; whereas, in Aguilera et al.'s study, at least two separate GC/C/IRMS analyses are required to measure the isotope ratio in the androstanediols and their

USADA 0827

ERC cholesterol acetate. However, in fairness, they did have a more difficult assay through the inclusion of testosterone itself among the analytes.

Manipulating the result of a drug test to give a "negative" result following testosterone administration would be difficult. Although the AD/PD ratio could be normalized by taking commercial pregnanediol (Sigma, $\delta^{13}C^0/00$ value −32.12) the low "absolute" value for pregnanediol would give the situation away. Similarly, commercial androstanediols also have very low $\delta^{13}C^0/00$ values (Sigma −34.00) so self-administration of these compounds would not increase the δ values of testosterone derived metabolites. Probably the only way to fool such a test would be to alter the diet drastically over a long period of time through exclusively ingesting foodstuffs with $\delta^{13}C^0/00$ values below −29. False positives could be obtained by ingestion of nonproscribed steroids such as the now commonly used dehydroepiandrosterone (DHEA), which also partially metabolizes to androstanediols. However, whether testosterone or DHEA was being administered could easily be determined from marked differences in the total urinary steroid profile.

Acknowledgments

These studies were made possible through the generous support of the U.S. Olympic Committee and its representatives Dr. Wade Exum and Dr. James Betts. Financial support was also provided by Children's Hospital Oakland Research Institute. The authors thank Dr. Victor Rogozkin (St. Petersburg), Dr. Lubos Stárka (Prague), and Drs. Xavier de la Torre and Jordi Segura (Barcelona) for donating synthetic testosterone standards. Thanks are also given to individuals from different countries who provided urine samples for analysis. These studies could not have been carried out without the continual mass spectrometric maintenance and training undertaken by Ms Esther Roitman.

References

1. Catlin DH, Cowan DA, De la Torre R, Donike M, Fraisse D, Oftegro H, Hatton CK, Starcevic B, Becchi M, de la Torre X, Norli H, Geyer H, Walker CJ (1996). Urinary testosterone (T) to epitestosterone (E) ratios by GC/MS. I. Initial comparison of uncorrected T/E in six international laboratories. *J Mass Spectrom* **31**:397–402.
2. Southan G, Mallet A, Jumeau J, Craig S, Poojara N, Mitchell D, Wheeler M, Brooks RV (1990). Programme and Abstracts of The 2nd International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona, Spain, p. 306.
3. Smith BN, Epstein S (1971). Two categories of $^{13}C/^{12}C$ ratios for higher plants. *Plant Physiol* **47**:380–384.
4. Becchi M, Aguilera R, Farizon Y, Flament IM-M, Casabianca H, James P (1994). Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rapid Commun Mass Spectrom* **8**:301–308.
5. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope JHG (1996). Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom* **31**:169–176.
6. Donike M, Bärwald KR, Klosterman K, Schänzer W, Zimmerman J (1983). *Sport.* Leistung und Gesundheit, Cologne, Germany, p. 293.
7. Shackleton CHL, Whitney JO (1980). Use of Sep-pak® cartridges for urinary steroids extraction: Evaluation of the method for use prior to gas chromatographic analysis. *Clin Chim Acta* **107**:231–243.
8. Girard A, Sandulesco G (1936). Sur une nouvelle série de réactif du groupe carbonyle, leur utilisation à l'extraction des substances cétoniques et à la characterisation microchinique des aldehydes et cétones. *Helv Chim Acta* **19**:1095–1107.
9. Bush IE (1961). *The Chromatography of Steroids.* Pergamon Press Ltd., Oxford, UK, pp. 358–371.
10. Setchell KDR, Shackleton CHL (1973). The group separation of plasma and urinary steroids by column chromatography on Sephadex LH-20. *Clin Chim Acta* **47**:381–388.
11. de la Torre X, Segura J, Farré M, Yang Z, Li Y, Wu M (1995). Steroid profile in different ethnic groups after testosterone ethanate administration. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke U (eds), *Recent Advances in Doping Analysis.* Sport und Buch Strauß, Köln, Germany, pp. 59–80.
12. Shackleton CHL, Honour JW (1976). Simultaneous estimation of urinary steroids by semiautomated gas chromatography. Investigation of neonatal infants and children with abnormal steroid synthesis. *Clin Chim Acta* **69**:267–283.

Journal of Chromatographic Science, Vol. 43, January 2005

Isotopic Fractionation of Endogenous Anabolic Androgenic Steroids and Its Relationship to Doping Control in Sports

Adam T. Cawley[1,2], Rymantas Kazlauskas[1,*], Graham J. Trout[1], Jill H. Rogerson[1], and Adrian V. George[2]

[1]Research Chemist, Australian Sports Drug Testing Laboratory, National Measurement Institute, 1 Suakin Street, Pymble, NSW 2073, Australia and [2]School of Chemistry, University of Sydney, NSW 2006, Australia

Abstract

The use of gas chromatography (GC)–combustion (C)–isotope ratio mass spectrometry (IRMS) demonstrates that a single oral administration of dehydroepiandrosterone (DHEA, 100 mg) to a male subject significantly lowers the ^{13}C content of etiocholanolone (Et) and androsterone (A) in the subject's urine. The difference in carbon isotope ratio ($\delta^{13}C$‰) values between Et and A increases from 1.6‰ at the time of administration to 5.1‰ at 26 h post-administration, indicating preferential metabolism of administered DHEA to form Et in relation to A. Multiple oral administrations of DHEA to a male subject reveals lower $\delta^{13}C$ values during the excretion period of Et (–31.7‰ to –34.6‰) and A (–31.4‰ to –33.0‰) to that of the $\delta^{13}C$ value of the administered DHEA (–31.3‰). Reference distributions of $\delta^{13}C$ Et and $\delta^{13}C$ A constructed from normal athlete populations within Australia and New Zealand show a small natural discrimination against ^{13}C in the formation of Et relative to A (mean = 0.3‰, $n = 167$, $p = 0.007$). Amplified differences between $\delta^{13}C$ Et and $\delta^{13}C$ A, and in vivo ^{13}C depletion measured by GC–C–IRMS are shown to be potentially useful for doping control.

Introduction

Abuse of endogenous (i.e., naturally occurring) steroids is one of the most important issues in sports. Doping control laboratories have been confronted with the challenge of finding criteria that allow endogenous steroids to be distinguished from their synthetic analogues in the urine of athletes. Dehydroepiandrosterone (DHEA) is an orally ingested weak endogenous androgen, sold in the United States as an over the counter "dietary supplement", which may be ingested by athletes with the aim of increasing levels of the more active androgens such as testosterone and dihydrotestosterone (1). The International Olympic Committee prohibits DHEA administration to an athlete (2), however the detection of administered DHEA by doping control laboratories has been difficult because of an incomplete understanding of DHEA metabolism as well as interindividual variations in urinary steroid excretion (3–9).

Attempts to detect DHEA abuse by gas chromatography (GC)–mass spectrometry (MS) alone have relied on the elevated excretion of the glucuronide conjugate of DHEA. Dehennin et al. (4) proposed that DHEA administration is indicated by a urinary concentration of DHEA–glucuronide exceeding the level of 300 ng/mL, based on reference studies showing the average normal concentration of DHEA–glucuronide to be less than 100 ng/mL. The usefulness of proposed limits such as this often depend on the metabolism of the individual athlete and the degree to which DHEA is converted to its sulfate conjugate (10). Obviously, more predominant conversion of DHEA to DHEA-sulfate will reduce the effectiveness of an administration marker based on DHEA–glucuronide. Previous studies by Kazlauskas (5) and Cawley (9) have found elevated DHEA–glucuronide excretions greater than 300 ng/mL for only 10 h after the administration of DHEA. Work by Ayotte et al. (6) has shown that DHEA administration resulted in increased excretion of C-7 hydroxylated metabolites: 7α- and 7β-hydroxy-DHEA. The detection of these compounds in the free/glucuronide steroid fraction routinely applied by doping control laboratories is difficult; however, because these two products are excreted as sulfate conjugates, which are not hydrolyzed by this process.

The use of GC–combustion (C)–isotope ratio MS (IRMS) analysis has been shown by various groups to be effective in detecting the administration of synthetic endogenous steroids (11–24). This is based on the principle of synthetic analogues of endogenous steroids, such as, DHEA, androstenedione, testosterone, and dihydrotestosterone having a considerably lower ^{13}C content compared with the naturally excreted compound (21). The GC–C–IRMS technique is capable of measuring these differences with a precision of 1‰ (25). A detailed description of the fundamental principles and nomenclature associated with the application of GC–C–IRMS analysis to doping control has been reported previously (26).

Most research to date has demonstrated the use of GC–C–IRMS

*Author to whom correspondence should be addressed.

nitrogen before the dry residue was dissolved in dichloromethane (100 µL) and transferred to a vial for GC–C–IRMS analysis.

GC–C–IRMS conditions

The system used was a Finnigan-MAT Delta Plus with a GC Combustion III from Thermo Finnigan (Bremen, Germany) for separation and online combustion of the steroid metabolites. The carrier gas was helium with a constant flow of 1.8 mL/min and initial pressure of 17.3 psi. The injection volume was 2 µL in splitless mode at 280°C with an interval of 0.5 min. The column (30–m × 25 –mm i.d.) was a Hewlett-Packard HP-50+ cross-linked 50% phenyl-methyl siloxane (0.25 µm film thickness). The column temperature was programmed from 180°C for 1 min to 250°C at 12°C/min, to 280°C at 3°C/min, then finally to 300°C at 15°C/min and held for 4 min. The combustion interface was used with an oxidation reactor temperature of 940°C. High purity oxygen gas was flushed through the furnace for 3600 s prior to analysis of a sequence. The reduction reactor temperature was 620°C. The software operating the IRMS system was ISODAT 7.4.

The $\delta^{13}C$ of Et, A, 11-ketoEt, and 17-MeT were determined in each of the urine samples from both administration studies. This required careful definition of each of the peaks relative to the background to ensure there was no systematic bias caused by erroneous integration. The $\delta^{13}C$ calculated by the software was relative to the calibrated value of $\delta^{13}C = -30.49‰$ that was determined by comparison with the primary standard, Vienna Pee Dee Belemnite (CSIRO, 1999). This value was set for the reference CO_2 peak defined by the same procedure as the steroid peaks. Stability and reproducability of GC–C–IRMS measurements was monitored by determining $\delta^{13}C$ 17-MeT in each sample that was always close to its value of –32.8‰ (CSIRO, 1999), measured by combustion ^{13}C analysis within a standard deviation of 0.7‰.

GC–MS analysis

Each sample extract analyzed by GC–C–IRMS was also analyzed by full-scan GC–MS under the same GC conditions previously outlined using a Hewlett-Packard HP 5890 GC coupled to a HP 5970 mass selective detector (MSD). This was performed in order to ensure spectral identification of A, Et, 11-ketoEt, and 17-MeT and verify the purity of these peaks. The MSD acquired data in scan mode from 40 to 450 amu and electron impact spectral comparison was made to standards run at the same time.

Table I. GC–C–IRMS Results From the Single Administration of DHEA to Subject A in Relation to Post-Administration Time

Sample	Time (h)	$\delta^{13}C$ (‰) Et	A	11-ketoEt	17-MeT
A0	0	–24.2	–22.6	–21.0	–32.5
A1	2	–25.7	–24.0	–20.9	–32.9
A2	5	–31.8	–30.0	–20.0	–33.3
A3	9	–32.1	–30.0	–19.3	–32.6
A4	11	–31.1	–28.6	–20.8	–32.8
A5	22	–31.4	–26.9	–21.1	–33.1
A6	26	–31.4	–26.3	–21.5	–33.5
A7	28	–30.8	–25.7	–20.4	–32.9
A8	30	–30.6	–25.7	–20.9	–33.7
A9	34	–30.1	–25.2	–20.6	–32.7
A10	41	–29.8	–25.1	–21.4	–32.9
A11	46	–29.3	–24.8	–21.9	–32.4
A12	48	–28.0	–24.1	–21.6	–32.3
A13	52	–26.3	–23.9	–21.6	–32.9

Reference population study

GC–C–IRMS analysis was conducted on a total of 167 urine samples collected from Australian ($n = 59$) and New Zealand ($n = 108$) athletes following approval of the Australian Institute of Sport Ethics Committee and the informed consent of each participant (30). Each of the athletes signed a guarantee that they had not ingested any substance prohibited by the Olympic Movement Anti-Doping Code (2), to ensure, as best as possible, that their urine samples could be used to represent a normal, drug-free population.

Results

The $\delta^{13}C$ of DHEA in the capsules administered to subjects A and B was determined to be –31.3‰ with a standard deviation of 0.5‰ obtained from triplicate IRMS analysis of duplicate extractions from two capsules (12 injections). A full-scan mass spectrum was collected of each DHEA sample to confirm the presence of DHEA in the administered capsules. The purity of the fraction

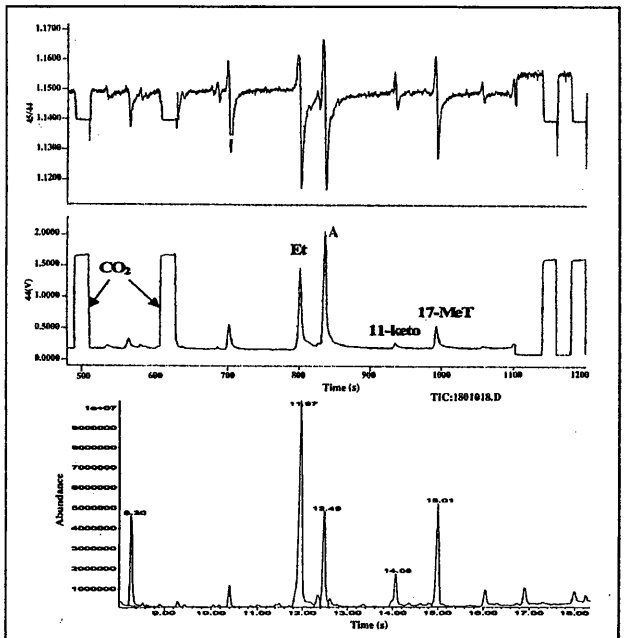


Figure 1. This shows the GC–C–IRMS trace for a sample (middle trace) at *m/z* 44 as well as the chromatogram for ratio of 45–44 masses (top trace). The large spikes are obtained from carbon dioxide, which is pulsed into the instrument several times as the reference against which the isotope ratios are measured. The etiocholanolone, androsterone, 11-ketoetiocholanolone, and the internal standard, 17-methyltestosterone are labeled. The GC–MS chromatogram (bottom trace), obtained under the same chromatographic conditions as the GC–C–IRMS, allows the identity of the IRMS peaks to be obtained by inspection of the mass spectrum obtained at the retention time of each peak.

administrations of DHEA to subject B shows that all samples after administration for the collection period would be found to be positive when applied to both $\delta^{13}C$ Et and $\delta^{13}C$ A values. The single DHEA administration to subject A shows that a post-administration detection period of 48 hours would result from a –27.0‰ criterion applied to $\delta^{13}C$ Et, although it would be much shorter (11 h) if applied $\delta^{13}C$ A. These changes are considerably longer than effects observed on metabolite concentrations as a means of detection of the parent substance (5,9).

Metabolic fractionation is illustrated (Figure 5) showing the $\delta^{13}C$ reference distribution of 11-ketoEt, the endogenous marker that is produced in a separate biosynthetic pathway to the androgens, to be ^{13}C-enriched relative to the $\delta^{13}C$ reference distributions of Et and A in the Australasian population. This is further highlighted in Figure 7, showing 99% of the population as having positive averaged differences. A consequence of this work finding larger-than-expected natural differences between the $\delta^{13}C$ values of Et and A relative to 11-ketoEt is that the proposed criteria (26) may need to be revised. Even though there were no samples in the Australasian reference population that had averaged differences greater than 4.0‰ (Figure 7), a 99.7% confidence level applied to this distribution revealed that the maximum expected value from athletes not doping may be as large as 4.8‰. The criteria would however, be supported by a 95% confidence level (i.e., mean plus two standard deviations) applied to this distribution. In relation to detecting DHEA abuse, averaged difference values remained far greater than 4.8‰ throughout the multiple administration study. In fact, the smallest difference observed was 8.7‰, which was recorded 22 h after the final administration.

The differences observed between $\delta^{13}C$ Et and $\delta^{13}C$ A produced from completely endogenous sources and those observed following DHEA administration can also be compared to propose analysis criteria. Statistical interpretation of the reference population suggests that a $\delta^{13}C$ Et–$\delta^{13}C$ A value greater than 3.7‰ could be used to indicate DHEA administration. This would also represent a 48-h post-administration detection period based on the single administration of DHEA to subject A.

Conclusion

This study demonstrated the use of GC–C–IRMS analysis of urinary steroid metabolites to provide more information on metabolic pathways following illegal steroid administration. Alteration in $\delta^{13}C$ values of A and Et in relation to each other showed a preference for prolonged formation of ^{13}C depleted Et from administered DHEA. Metabolism of orally administered DHEA, with respect to the proportions forming Et and A, appears to be related to pharmacokinetic mechanisms resulting from excess DHEA in the body. Furthermore, it displays significant differences to that of endogenous DHEA. The complex nature of steroid metabolism in the body was also highlighted following multiple administrations of DHEA, showing in vivo ^{13}C depletion of androgen metabolites. Comparison of reference values obtained from a normal population with those obtained from administration studies allowed several criteria to be proposed for GC–C–IRMS analysis in doping control. This includes the absolute values of Et and A, use of an endogenous marker representing reference values that is a necessary component of any doping control process, and changes in the values of metabolites reflecting kinetic isotopic fractionation ($\delta^{13}C$ Et–$\delta^{13}C$ A). It is envisaged that, in the future, such measurements as a combinatorial factor will provide protocols that are valuable for confirming the administration of synthetic analogues of particular endogenous steroids.

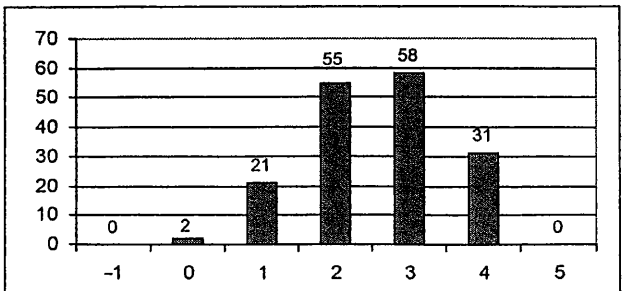


Figure 7. Frequency distribution of the difference between the average $\delta^{13}C$ value of Et and A and $\delta^{13}C$ 11-ketoEt in the Australasian population of 167 samples. This shows that there were 31 samples with values between 3.0‰ and 4.0‰ but no samples with values greater than 4.0‰.

Acknowledgments

We would like to thank Dr. Robert Weatherby, Senior Lecturer at the School of Exercise Science and Sport Management at Southern Cross University (Lismore, NSW, Australia), for providing the administration trials for this study. Special mention must also go to Mrs. Kishwar Rahman from ASDTL for her important work in extracting the 167 urine samples used in the reference population. The Australian Commonwealth Government through the Sport and Recreation (anti-doping) Program of the Department of Communications, Information Technology and the Arts supported the presentation of reference populations in this paper. We also acknowledge the financial support provided to the development of GC–C–IRMS analysis in doping control by the Olympic Research Program jointly funded by the Sydney Organising Committee for the Olympic Games and the Australian Commonwealth Government.

References

1. B. Corrigan (1999). Dehydroepiandrosterone and sport. Medical Journal of Australia, Sydney, Australia. http://www.mja.com.au/public/issues/171_4_160899/corrigan/corrigan.html (accessed July 29, 2003).
2. Olympic Movement Anti-Doping Code, International Olympic Committee (1999). http://multimedia.olympic.org/pdf/en_report_21.pdf (accessed July 29, 2003).
3. T.Z. Bosy, K.A. Moore, and A. Poklis. The effect of oral dehydroepiandrosterone (DHEA) on the urine testosterone/epitestosterone (T/E) ratio in human male volunteers. *J. Anal. Toxicol.* **22:** 455–59 (1998).
4. L. Dehennin, M. Ferry, P. Lafarge, G. Pérès, and J.-P. Lafarge. Oral administration of dehydroepiandrosterone to healthy men: alteration

of the urinary androgen profile and consequences for the detection of abuse in sport by gas chromatography-mass spectrometry. *Steroids* **63:** 80–7 (1998).
5. R. Kazlauskas. "Effects of dehydroepiandrosterone on urinary steroids". In *Recent Advances in Doping Analysis* (5). W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Cologne, Germany, 1998, pp. 83–90.
6. J.F. Lévesque and C. Ayotte. "The oral administration of DHEA: the efficiency of steroid profiling". In *Recent Advances in Doping Analysis* (7). W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Cologne, Germany, 1999, pp. 213–15.
7. L.D. Bowers. Oral dehydroepiandrosterone supplementation can increase the T/E ratio. *Clin. Chem.* **45:** 295–97 (1999).
8. V.P. Uralets and P.A. Gillette. Over-the-counter Δ^5 anabolic steroids 5-androsten-3,17-dione; 5-androsten-3β,17β-diol; dehydroepiandrosterone; and 19-nor-5-androsten-3,17-dione: excretion studies in men. *J. Anal. Toxicol.* **24:** 188–93 (2000).
9. A.T. Cawley. The detection of dehydroepiandrosterone administration in athletes by GC-MS and GC-C-IRMS. *Honours thesis.* The University of Sydney, Sydney, Australia 2001, p. 18.
10. J.B. Adams. Control of the secretion and the function of C_{19}-Δ^5-steroids of the human adrenal gland. *Molecular and Cell Endocrinology* **41:** 1–17 (1985).
11. M. Becchi, R. Aguilera, Y. Farizon, M-M. Flament, H. Casabianca, and P. James. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect abuse of testosterone in sport. *Rapid Commun. Mass Spectrom.* **8:** 304–308 (1994).
12. R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic, and H.G. Pope, Jr. Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom.* **31:** 169–76 (1996).
13. S. Horning, H. Geyer, M. Machnik, W. Schänzer, A. Hilkert, and J. Oesselmann. "Detection of exogenous testosterone by $^{13}C/^{12}C$ analysis". In *Recent Advances in Doping Analysis* (4). W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Cologne, Germany, 1997, pp. 275–83.
14. C.H.L. Shackleton, E. Roitman, A. Phillips, T. Chang, and Y. Li. Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. *Steroids* **62:** 379–87 (1997).
15. C.H.L. Shackleton, E. Roitman, A. Phillips, and T. Chang. Androstanediol and 5-androstenediol profiling for exogenously administered dihydrotestosterone, epitestosterone and dehydroepiandrosterone: potential use in gas chromatography isotope ratio mass spectrometry. *Steroids* **62:** 665–73 (1997).
16. S. Horning, H. Geyer, U. Flenker, and W. Schänzer. "Detection of exogenous steroids by $^{13}C/^{12}C$ analysis". In Recent Advances in Doping Analysis (5). W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Cologne, Germany, 1998, pp. 135–48.
17. U. Flenker, S. Horning, E. Nolteernsting, H. Geyer, and W. Schänzer. "Measurement of $^{13}C/^{12}C$-ratios to confirm misuse of endogenous steroids". In *Recent Advances in Doping Analysis* (6). W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Cologne, Germany, 1999, pp. 243–56.
18. R. Aguilera, D.H. Catlin, M. Becchi, A. Phillips, C. Wang, R.S. Swerdloff, H.G. Pope, and C.K. Hatton. Screening urine for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. *J. Chromatogr. B Biomed. Sci. Appl.* **727:** 95–105 (1999).
19. J. Segura, S.H. Peng, and X. de la Torre. Recent progress in the detection of the administration of natural hormones: special focus on testosterone. *J. Toxicol. –Toxin Reviews* **18:** 125–44 (1999).
20. M. Ueki and M. Okano. Doping with naturally occurring steroids. *J. Toxicol. –Toxin Reviews* **18:** 177–95 (1999).
21. M. Ueki and M. Okano. Analysis of exogenous dehydroepiandrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **13:** *2237–43 (1999).*
22. R. Aguilera, T.E. Chapman, and D.H. Catlin. A rapid screening assay for measuring urinary androsterone and etiocholanolone $\delta^{13}C$ (‰) values by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **14:** 2294–99 (2000).
23. C. Ayotte, D. Goudreault, A. Lajeunesse, M. Cléroux, Y. Richard, A. Charlebois, J.-P. Couture, and A. Fakirian. "GC/C/IRMS and GC/MS in natural steroids testing". In *Recent Advances in Doping Analysis* (9). W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Cologne, Germany, 2001, pp. 133–43.
24. M. Ueki, T. Takao, M. Okano, A. Ikekita, and M. Sato. Differentiation of endogenous and exogenous steroids in urine by means of isotope ratio mass spectrometry. In *Recent Advances in Doping Analysis* (9). W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Cologne, Germany, 2001, pp. 145–54.
25. W.A. Brand. High precision isotope ratio monitoring techniques in mass spectrometry. *J. Mass Spectrom.* **31:** 225–35 (1996).
26. G.J. Trout, J.H. Rogerson, A.T. Cawley, and C.W. Alma. Developments in sports drug testing. *Aust. J. Chem.* **56:** 175–80 (2003).
27. J.H. Rogerson, G.J. Trout, and R. Kazlauskas. Personal communication. Australian Sports Drug Testing Laboratory, 2000.
28. D.B. Gower. "Catabolism and excretion of steroids". In *Biochemistry of steroid hormones.* H.L.J. Makin, Ed. Blackwell Scientific Publications, London, U.K. 1975, pp. 149–64.
29. Human Ethics Committee of Southern Cross University. Studies on the pharmacokinetics of DHEA and related substances–1998. Lismore, NSW, Australia, approval number ECN-98-42.
30. Australian Institute of Sport Ethics Committee. Development of reference ranges for blood and urine parameters in elite athletes. Canberra ACT. Australia, approval date 21/9/1999.
31. U. Flenker and W. Schänzer. "Kinetic isotope effects during metabolism of Δ^4-steroids". In *Recent Advances in Doping Analysis* (9). W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Cologne, Germany, 2001, pp. 179–85.
32. W. Schänzer. Metabolism of anabolic androgenic steroids. *Clin. Chem.* **42:** 1001–20 (1996).
33. S.C. Cunnane and S.S. Likhodil. ^{13}C NMR spectroscopy and gas chromatograph-combustion-isotope ratio mass spectrometry: complementary applications in monitoring the metabolism of ^{13}C-labelled polyunsaturated fatty acids. *Can. J. Physiol. Pharmacol.* **74:** 761–68 (1996).

Manuscript accepted August 3, 2004.

Document Number:	TD2004EAAS	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Date:	30 May, 2004	Effective Date:	13 August, 2004

REPORTING AND EVALUATION GUIDANCE FOR TESTOSTERONE, EPITESTOSTERONE, T/E RATIO AND OTHER ENDOGENOUS STEROIDS

1. Introduction:

This guide has been prepared to ensure that Laboratories can report, in a uniform way, the presence of abnormal profiles of urinary steroids resulting from the administration of testosterone or its precursors, androstenediol, androstenedione, dehydroepiandrosterone (DHEA) or a testosterone metabolite, dihydrotestosterone or a masking agent, epitestosterone. It also provides guidance to the Testing Authority on how to conduct the evaluation of *Adverse Analytical Findings* reported by the Laboratories.

It is proven that administration of these steroids alters one or more of the parameters of the urinary steroid profile. Elevated levels of urinary metabolites, which are part of the "steroid profile", e.g. testosterone, epitestosterone, dihydrotestosterone, androsterone, etiocholanolone, DHEA as well as other specific metabolites are not consistent with normal endogenous production and result from the intake of these steroids. Increased ratios of specific pairs of steroid metabolites are also indicative of the administration of these endogenous steroids.

It is emphasized that the following requirements shall be applied by all Laboratories in their routine practice.

2. Specific requirements for GC/MS measurement of T/E value, concentration of testosterone, concentration of epitestosterone:

The T/E value is given by the peak area or peak height ratio of testosterone and epitestosterone (equivalent to the glucuronide) obtained by measuring the ion at m/z 432 by GC/MS analysis in a Single Ion Monitoring mode (SIM). The T/E value is usually measurable regardless of the concentration of both steroids. Whether measured from the Screening Procedure or the Confirmation Procedure, it must be corrected using an appropriate standard (e.g. calibration curve, quality control sample(s) or authentic standard solutions of both testosterone and epitestosterone). The concentration of testosterone and epitestosterone (equivalent to the glucuronide) should be estimated but should not be used to determine the T/E value. In the case of high T/E values, the concentration of epitestosterone is frequently low and it may not always be possible to measure epitestosterone precisely. In such cases, only the concentration of testosterone (equivalent to the glucuronide) is to be determined.

The Screening Procedure which is normally conducted on a single aliquot shall be carried out including, together in the same batch, a control sample where the T/E value, concentrations of testosterone and epitestosterone are known.

Reference ranges of the various parameters of the urinary steroid profile have been described for populations of both males and females. It should be borne in mind that there is significant

variation between individuals . A normal level for one individual may in another be elevated and be consistent with doping. The Laboratory will adapt its testing procedures to the *Sample* tested; for example, female or male, Asian or Caucasian (when the information is provided). The concentration of urinary steroids such as testosterone and epitestosterone varies greatly between individuals and also depends upon the specific gravity of the urine *Sample*; only values corrected for a specific gravity value of 1.020 can be compared.

It is recommended that a urine *Sample* in which any one of the following criteria is met during the Screening Procedure, be routinely submitted to the IRMS analysis:

i) T/E value equal or greater than 4;
ii) concentration of testosterone or epitestosterone (equivalent to the glucuronide) greater than 200 ng/mL[1];
iii) concentration of androsterone or etiocholanolone (equivalent to the glucuronide) greater than 10,000 ng/mL[1];
iv) concentration of DHEA (equivalent to the glucuronide) greater than 100 ng/mL[1].

It is recognised that other parameters may justify a need for IRMS study and the reason should be documented.

Any result that will be used to support an *Adverse Analytical Finding* shall be confirmed and quantified.

Confirmation of elevated T/E values, concentration of testosterone, epitestosterone or any other steroid metabolite under consideration is to be performed in triplicate. The confirmation of the identity of any steroid reported with abnormal properties must be made (refer to technical document TD2003IDCR). Appropriate calibration (e.g. calibration curve, deuterated standards, quality control samples) is to be included in the protocol of the Confirmation Procedure.

Confirmed elevated concentration of steroids will be reported as such together with the value adjusted for the specific gravity of the urine *Sample* using the following formula:

$$\text{Concentration}_{1.020}\ \text{ng/mL} = (1.020 - 1) / (\text{Specific gravity of the } \textit{Sample} - 1) \bullet \text{Concentration measured ng/mL}$$

The urine *Sample* is not collected under sterile conditions, and where the circumstances are favourable, the microbes present in the *Sample* can cause changes to the profile of the urinary steroids. Initially there is cleavage of the glucuronides and sulfates followed by modifications of the steroids' structure by oxido-reductive reactions. To report an *Adverse Analytical Finding* of an elevated T/E value, testosterone or epitestosterone concentration or any other endogenous steroid parameters, the concentration of free testosterone and/or epitestosterone in the specimen is not to exceed 5% of the respective glucuroconjugates. Elevated amounts of 5α- and 5ß-androstan-3,17-dione in the free form also indicate microbial degradation.

[1] Concentrations adjusted for a specific gravity value of 1.020

3. Isotope ratio mass spectrometry:

When a parameter of the steroid profile indicates a need to further study, its $^{13}C/^{12}C$ value expressed in delta units per mil (d $^{0}/_{00}$) or that of its metabolites will be measured and compared to that of urinary reference steroids within the sample not affected by administration. Depending upon the nature of the endogenous steroid suspected to have been administered, the metabolites analysed could be testosterone, epitestosterone, androsterone, etiocholanolone, the androstanediols, DHEA, or other relevant metabolites while the urinary reference steroid usually analysed by the Laboratories is one of, pregnanediol, pregnanetriol, cholesterol, 11-hydroxyandrosterone or 11-ketoetiocholanolone. The instrumentation should be calibrated with an appropriate Reference Material.

The results will be reported as consistent with the administration of a steroid when the $^{13}C/^{12}C$ value measured for the metabolite(s) differs significantly i.e. by 3 delta units or more from that of the urinary reference steroid chosen. In some *Samples*, the measure of the $^{13}C/^{12}C$ value of the urinary reference steroid(s) may not be possible due to their low concentration. The results of such analyses will be reported as "inconclusive" unless the ratio measured for the metabolite(s) is below -28‰ based on non-derivatised steroid.

4. Reviewing and evaluating test results:

The following actions should be requested by the Testing Authority in agreement with the Laboratory:

- Isotopic ratios ($^{13}C/^{12}C$) of the relevant metabolites should whenever possible be measured each time an elevated parameter of the steroid profile is estimated from the Screening Procedure or Confirmation Procedure and reported to the Testing Authority as having been determined. If the Laboratory does not have the capability to conduct such testing, the *Samples* are to be securely transferred ensuring the Chain of Custody to another Laboratory with the requisite capability.

- The results of the IRMS analysis and/or of the steroid profile measured by GC/MS shall be used to draw conclusions as to whether a doping violation may have been committed. If the IRMS study does not readily indicate exogenous administration, the result should be reported as "inconclusive" and if necessary further longitudinal studies performed.

- When available, the athlete's previous tests on record at the Testing Authority should be accessed and the corresponding steroid profile data requested from the relevant Laboratory. These results should be examined and considered together with the existing evidence (longitudinal study).

- If, for any reason, an IRMS analysis cannot be carried out satisfactorily (e.g. insufficient volume of urine, amount of analyte too low to enable a valid measurement) or the examination of previous test results raises suspicions due to unstable profile values, up to three further unannounced tests should be carried out, preferably within a three months period following the report of the suspicious analytical result. There should be a minimum total of three results, other than the abnormal *Sample*, of either past or post data. A *Sample* in which the elevated parameter is again measured is to be analysed by IRMS as described above. In difficult cases longer monitoring may be required.

5. Evaluation of longitudinal studies:

In males, the individual T/E values have been shown to vary from their mean value by less than 30% (screening values). In females, a low concentration of some urinary steroids such as epitestosterone and testosterone, close to the limit of detection using current analytical methods occurs. Normal variation of up to 60% may be expected. The individual basal T/E value should be determined from at least three test results, excluding the suspicious result under consideration. The mean, standard deviation and coefficient of variation (expressed in percent) should be calculated for those three basal values. If the suspicious test result, when compared to the basal value using appropriate statistical evaluation is found to be significantly different, that will constitute a proof of the administration of a source of testosterone. It is understood that the basal value may be calculated from previous screening test results. The comparison of screening results and confirmed results is acceptable.

The same reasoning applies to any other parameter of the steroid profile which has been estimated to be in an amount exceeding the ranges of values normally found in humans.

6. Other parameters:

Other parameters such as the ratio of urinary testosterone to Lutenising Hormone (T/LH) and the androsterone to testosterone ratio (A/T) may be used to provide extra information to help determine the use of some substances especially injected testosterone and many of its esters. A high T/LH ratio may be used as ancillary evidence. The A/T ratio which has markedly changed from the "normal" value found for an individual during a longitudinal study may indicate which type of substance has been used. A change to high value can indicate testosterone use and a change to low values may indicate the use of testosterone precursors such as DHEA. However, any administration of testosterone and of its precursors, androstenedione or DHEA will not necessarily alter the excretion of LH and epitestosterone glucuronide.

7. Examples of specific urinary metabolites potentially altered by the administration of "endogenous steroids";

Urinary steroid	Steroid administered
Testosterone (G)	Testosterone, androstenedione, DHEA
Epitestosterone (G)	Epitestosterone
T/E (G)	Testosterone, androstenedione, DHEA
Androsterone (G)	Testosterone, DHT, androstenedione, DHEA and androstenediol
Etiocholanolone (G)	Testosterone, androstenedione, DHEA and androstenediol
DHEA (G) (S)	DHEA
6a-OH Androstenedione (G)	Androstenedione
6β-OH Androsterone (G)	Androstenedione
6β-OH Etiocholanolone(G)	Androstenedione
6β-OH Epiandrosterone (S)	Androstenedione
7β-OH DHEA/16α-OH Androsterone (S)	DHEA
7-OH DHEA, 7 keto DHEA	7 keto DHEA

* *G indicates the glucuronide and S indicates sulphate conjugation.*

The official text of the technical document Reporting and Evaluation Guidance for Testosterone, Epitestosterone, T/E Ratio and other Endogenous Steroids shall be maintained by *WADA* and shall be published in English and French. In the event of any conflict between the English and French versions, the English version shall prevail.

8. References:

Aguilera R., M. Becchi, H. Casabianca, C.K. Hatton, D. H. Catlin and B. Starcevic, *Improved method of detection of testosterone abuse by gas chromatography /combustion/ isotope ratio mass spectrometry analysis of urinary steroids*, J. Mass Spectrom. 31 (1996) 169

Aguilera, R., Catlin, D.H., Becchi, M., Phillips, A., Wang, C., Swerdloff, R.S., Pope, H.G., and Hatton, C.K. (1999). *Screening for exogenous testosterone by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols.* J. Chromatogr.B. 727:95.

Aguilera, R., Chapman, T.E., and Catlin, D.H. (2000). *A rapid screening assay for measuring urinary androsterone and etiocholanolone* $\delta^{13}C$ ($^{0}/_{00}$) *values by gas chromatography/combustion/isotope ratio mass spectrometry.* Rapid Commun. Mass Spectrom.14: 2294.

Aguilera, R., Chapman, T.E., Starcevic, B., Hatton, C.K., and Catlin, D.H. (2001). *Performance characteristics of a carbon isotope ratio method for detecting doping with testosterone based on*

urine diols: controls and athletes with elevated testosterone/epitestosterone ratios. Clin. Chem. 47: 292.

Aguilera R, Hatton CK, Catlin DH, *Detection of epitestosterone doping by isotope ratio mass spectrometry.*Clin Chem. 2002;48(4):629-36.

Ayotte C., Charlebois, A., Lapointe, S., Barriault D. and Sylvestre M. : *Validity of urine samples : microbial degradation. Recent advances in doping analysis*. Proceedings of the 14th Köln Workshop on Dope Analysis, Institut für Biochemie, Köln, Germany, March 17-22, 1996, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln, pp 127-137 (1997).

Ayotte C., D. Goudreault, A. Lajeunesse, M. Cléroux, Y. Richard, A. Charlebois, J. -P. Couture and A. Fakirian: *GC/C/IRMS and GC/MS in "Natural" Steroids Testing*, in Recent Advances in Doping Analysis (9), Proceedings of the 19th Cologne Workshop on Dope Analysis, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln (2001) p. 133

Ayotte C., *Evaluation of elevated testosterone/epitestosterone values in athletes' urine samples*, IAF New Studies in Athletics, 12:2-3 (1997) p. 87 and reference cited

Ayotte C.: *The integrity of urine samples: the effects of temperature and bacteria on the validity of the analytical result*. Proceedings of the IAF Seminar 1995, Paris, France, October 10-11, 1995, in " Harmonisation of Doping Issues in the IAAF ", p. 127-138 (1997)

Baenziger J. and L. Bowers, *Variability of T/E ratios in Athletes*, Proceedings of the 11th Cologne Workshop on dope analysis, 7th to 12th March 1993, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth eds, Sport und Buch Strausse Edition Sport, Koln (1994) p.41

Bassindale T., D.A Cowan, S. Dale, A.J. Hutt, A.R. Leeds and A.T. Kicman, *Disposition of androstenedione and testosterone following oral administration of androstenedione to healthy female volunteers: influence on the urinary T/E ratio*, in Recent Advances in Doping Analysis (10), Proceedings of the 20th Cologne Workshop on Dope Analysis, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln (2002) p. 51

Becchi M., R. Aguilera, Y. Farizon, M. M. Flament, H. Casabianca and P. James, *Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport*, Rapid Commun. Mass Spectrom., 8 (1994) 304

Bosy, T.Z., Moore, K.A., and Poklis, A. (1998). *The effect of oral dehydroepiandrosterone (DHEA) on the urine testosterone/epitestosterone (T/E) ratio in human male volunteers*. J. Anal. Toxicol. 22 (6): 455.

Bowers, L.D. (1999). *Oral Dehydroepiandrosterone supplementation can increase the testosterone /epitestosterone ratio*. Clin. Chem. 45 (2): 295.

Carlstrom K., E. Palonek, M. Garle, H. Oftebro, J. Stanghelle and I. Bjorkhem, *Detection of testosterone administration by increased ratio between serum concentrations of testosterone and 17 alpha-hydroxyprogesterone*, Clin. Chem., 38 (1992) 1779

Catlin D. H., C. K. Hatton and S. H. Starcevic, *Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athlete's urine,* Doping in Sport Symposium, Clin.Chem.,43(7) (1997) 1280

Catlin D. H., C. K. Hatton, P. W. Straus and B. Starcevic, in D. A. Cowan and A. T. Kicman (eds), *Control of Doping with Anabolic Agents*, Proceedings of the Scientific Meeting of the 4th Permanent world Conference on Anti-Doping in Sport, London (1993) p. 2

Catlin D.H., Leder BZ, Ahrens BD, Hatton CK, Finkelstein JS. *Effects of androstenedione administration on epitestosterone metabolism in men* Steroids. 2002 Jun;67(7):559-64.

Cowan D. A., A. T. Kicman, C. J. Walker and M. J. Wheeler, *Effect of administration of human chorionic gonadotrophin on criteria used to assess testosterone administration in athletes*, J. Endocrinol., 131 (1991) 147

Dehennin L. and A. M. Masumoto, *Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport*, J. Steroid Biochem. Mol. Biol., 44 (1993) 179

Donike M., H. Geyer, M. Kraft and S. Rauth, *Long-term Influence of Anabolic Steroid Misuse on the Steroid Profile*, in P. Belotti, G. Benzi, A. Ljungqvist eds., Doping in Sport, Monte Carlo 1989, International Athletic Foundation, Monte Carlo (1990) 107.

Donike M., K.-R. Barwald, K. Klostermann, W. Schanzer and J. Zimmermann, *Nachweis von exogenem Testosteron in Sport : Leistung und Gesundheit*, H. Heck, W. Hollmann, H. Liesen, R. Rost eds, Deutscher Arzte Verlag Koln, (1983) 293;

Donike M., S. Rauth and A. Wolansky, *Reference Ranges of urinary endogenous steroids determined by GC/MS*, Proceedings of the 10th Cologne Workshop on dope analysis, 7th to 12th June 1992, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth eds, Sport und Buch Strausse Edition Sport, Koln (1993) p.69

Donike M., S. Rauth, U. Mareck-Engelke, H. Geyer and R. Nitschke, *Evaluation of longitudinal studies, the determination of subject based reference ranges of the T/E ratio*, Proceedings of the 11th Cologne Workshop on dope analysis, 7th to 12th March 1993, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth eds, Sport und Buch Strausse Edition Sport, Koln (1994) p.33

Donike M., *Steroid Profile in Cologne*, Proceedings of the 10th Cologne Workshop on Dope Analysis, 7th to 12th June 1992, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth eds, Sport und Buch Strausse Edition Sport, Koln (1993) p.47

Donike M., U. Mareck-Engelke and S. Rauth, *Statistical evaluation of longitudinal studies, part 2 : the usefulness of subject based reference ranges*, Proceedings of the 12th Cologne Workshop on Dope analysis, Koln 1995 p. 157

Falk O., E. Palonek and I. Bjorkhem, *Effect of Ethanol on the Ratio between testosterone and epitestosterone in urine*, Clin. Chem., 34(7) (1988) 1462

Flenker U. and Schanzer W., *Kinetic isotope effects during metabolism of delta-4-steroids,* in Recent Advances in Doping Analysis (9), Proceedings of the 19th Cologne Workshop on Dope Analysis, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln (2001) p. 179

Leder BZ, Catlin DH, Longcope C, Ahrens B, Schoenfeld DA, Finkelstein JS. *Metabolism of orally administered androstenedione in young men.*J Clin Endocrinol Metab. 2001 Aug;86(8):3654-8.

Garle M., R. Ocka, E. Palonek and I. Bjorkhem, *Increased urinary testosterone/epitestosterone ratios found in Swedish athletes in connection with a national control program. Evaluation of 28 cases,* J. Chromatogr. B, 687 (1996) 55

Garle, M., and Palonek, E. (1998). *Androstenedione: excretion studies from single and multiple dose experiments.* In: Recent advances in doping analysis (6), Proceedings of the Manfred Donike Workshop, 16th Cologne Workshop on Dope Analysis, 15th to 20th March 1998, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke, (Eds,), Verlag Sport und Buch Straβ, Edition Sport, p. 181. Koln, Germany.

Geyer H., U. Mareck-Engelke, W. Schanzer and M. Donike, *The Cologne protocol to follow up high testosterone/epitestosterone ratios*, in Recent advances in doping analysis (4), Proceedings of the 14th Cologne Workshop on dope analysis, 12th to 17th March 1996, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, eds, Sport und Buch Strausse Edition Sport, Koln (1997) p.139

Geyer H., W. Schanzer, U. Mareck-Engelke and M. Donike, *Factors Influencing the Steroid Profile,* in Recent advances in doping analysis (3), Proceedings of the 13th Cologne Workshop on dope analysis, 12th to 17th March 1995, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, eds, Sport und Buch Strausse Edition Sport, Koln (1996) p.95

Hemmersbach P., K. I. Birkeland, J. R. Norli and S. H. Ringertz, *Urine Storage Conditions and Steroid Profile Analysis,* in Recent Advances in Doping Analysis (4), Proceedings of the 14th Cologne Workshop on Dope analysis, 12th to 17th March 1996, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, eds, Sport und Buch Strausse Edition Sport, Koln (1997) p.99

Horning S., H. Geyer, M. Machnik, W. Schanzer, A. Hilkert and J. Oeβelmann, *Detection of Exogenous testosterone by $^{13}C/^{12}C$ Analysis*, in Recent Advances in Doping Analysis (4),

Proceedings of the Manfred Donike Workshop 14th Cologne Workshop on Dope Analysis, 17th to 22nd March 1996, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, eds, Sport und Buch Strausse Edition Sport, Koln (1997) p. 275

Kazlauskas, R., *Effects of DHEA on urinary steroids,* in Recent Advances in Doping Analysis (5), Proceedings of Koln Workshop, (1997) p. 83

Kicman A. T., H. Oftebro, C. Walker, N. Norman and D. A. Cowan, *Potential use of ketoconazole in a dynamic endocrine test to differentiate between biological outliers and testosterone use by athletes,* Clin. Chem., 39 (1993) 1798

Kicman A. T., R. V. Brooks, S. C. Collyer, D. A. Cowan, M. N. Nanjee, G. H. Southan and M. J. Wheeler, *Criteria to indicate testosterone administration*, Br. J. Sport Med., 24 (1991) 253

Lévesque J.-F. and Ayotte C. : *The oral administration of DHEA : the efficiency of steroid profiling*, in Recent Advances in Doping Analysis (7), Proceedings of the 17th Cologne Workshop on Dope Analysis, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln (1999) p. 213

Lévesque J.-F. and Ayotte, C. : *Criteria for the detection of Androstenedione oral administration*, in Recent Advances in Doping Analysis (7), Proceedings of the 17th Cologne Workshop on Dope Analysis, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln (1999) p. 169

Liu X., Zhang Y., Wu M. and Zhang C., *The steroid profiles after oral administration of testosterone undecanoate with different doses*, in Recent Advances in Doping Analysis (7), Proceedings of the 17th Cologne Workshop on Dope Analysis, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln (1999) p. 311

Mareck-Engelke U., H. Geyer and M. Donike, *Stability of Steroid profiles*, Proceedings of the 10th Cologne Workshop on dope analysis, 7th to 12th June 1992, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth eds, Sport und Buch Strausse Edition Sport, Koln (1993) p.87

Mareck-Engelke U., H. Geyer and M. Donike, *Stability of Steroid profiles (4) : The circadian rhythm of urinary ratios and excretion rates of endogenous steroids in female and its menstrual dependency*, in Recent advances in doping analysis (2), Proceedings of the 12th Cologne Workshop on dope analysis, 10th to 15th April 1994, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, eds, Sport und Buch Strausse Edition Sport, Koln (1995) p.135;

Mareck-Engelke U., H. Geyer and M. Donike, *Stability of steroid profile (2) : excretion rates from morning urines*, Proceedings of the 11th Cologne Workshop on Dope analysis, Koln 1994 p. 85

Mareck-Engelke U., H. Geyer and M. Donike, *Stability of steroid profiles (3) : ratios and excretion rates of endogenous steroids in male urines collected over 24 hours*, Proceedings of the 12th Cologne Workshop on Dope analysis, Koln 1995 p. 121

Mareck-Engelke U., H. Geyer, U. Schindler, U. Flenker, R. Iffland and M. Donike, *Influence of Ethanol on Steroid Profile Parameters*, in Recent Advances in Doping analysis (3), Proceedings of the 13th Workshop on Dope Analysis, 12th to 17th March 1995, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, Sport und Buch Strau? ?dition Sport, Koln (1996) p.143

Mareck-Engelke U., U. Flenker and M. Donike, *Stability of Steroid profiles (5) : The annual rhythm of urinary ratios and excretion rates of endogenous steroids in female and its menstrual dependency*, in Recent advances in doping analysis (3), Proceedings of the 13th Cologne Workshop on dope analysis, 12th to 17th March 1995, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, eds, Sport und Buch Strausse Edition Sport, Koln (1996) p.177

Mareck-Engelke U., U. Flenker and W. Schanzer, *Stability of Steroid profiles (6) : The influence of oral contraceptives on steroid profiles*, in Recent advances in doping analysis (4), Proceedings of the 14th Cologne Workshop on dope analysis, 12th to 17th March 1996, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, eds, Sport und Buch Strausse Edition Sport, Koln (1997) p.139

Namba O., Y. Miyachi, M. Irie and Y. Kuroda, *Urinary testosterone and epitestosterone secretion in a doping positive subject*, International Congress of Endocrinology, Kyoto, 1988, Abstract 16-22-324

Palonek E. and M. Garle, *Single Injection of Testosterone to 7 Volunteers : Results from this Study*, in Proceedings of the 10th Cologne Workshop on Dope analysis, M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth (eds), Sport und Buch Strausse, Edition Sport, Koln, 1993, p. 131

Raynaud E., M. Audran, J. F. Brun, C. Fedou, J. L. Chanal and a. Orsetti, *False-positive cases in detection of testosterone doping*, Lancet (Letter), 340 (1992) 1468

Schweizer C., C. Cardis, M. Cauderay, L. Rivier and M. Saugy, *T/E ratio variations through puberty in male adolescents*, Proceedings of the 14th Cologne Workshop on Dope analysis, Koln 1997 p. 159

Setiawati E., Suyatna F.D., Wiria, M.S. and Ulamsari D., *Profile of urinary excretion in Indonesian volunteers after DHEA administration*, in Recent Advances in Doping Analysis (9), Proceedings of the 19th Cologne Workshop on Dope Analysis, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln (2001) p. 287

Shackleton, C.H.L., Phillips, A., Chang, T., and Li, Y. (1997). *Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols*. Steroids, 62: 379.

Shackleton, C.H.L., Roitman, E., Phillips, A., and Chang, T. (1997). *Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: Potential use in gas chromatography isotope ratio mass spectrometry*. Steroids, 62: 665.

Ueki, M., and Okano, M. (1999). *Analysis of exogenous dehydroepiandrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry*. Rapid Commun. Mass Spectrom. 13: 2237.

Uralets V. P., Gillette P.A. and Latven R.K., *Over-the-counter anabolic steroids 4-androsten-3,17-dione, 4-androsten-3,17-diol and 19-Nor-4-androsten-3,17-dione : excretion studies in men*, Recent advance in doping analysis (6), Proceedings of the Manfred Donike Workshop, 16th Cologne Workshop on Dope Analysis, 15th to 20th March 1998,W. Schanzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke (eds.), Sport & Buch Strauss (1999) p. 147 ;

Uralets, V.P., and Gillette, P.A. (1999). *Over-the-Counter anabolic steroids 4-androsten-3,17-dione; 4-androsten-3,17-diol and 19-nor-4-androsten-3,17-dione: excretion studies in men*. J. Anal. Toxicol., 23: 357.

Uralets V.P. and Gillette P.A. (2002). *New anabolic steroids available as nutritional supplements: 5-alpha-androstan-3,17-diol, 1,4-androstadien-3,17-dione and 5 alpha-androst-1-en-17-ol-3-one*, in Recent Advances in Doping Analysis (10), Proceedings of the 20th Cologne Workshop on Dope Analysis, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln (2002) p. 73

Van Eenoo, P., Delbeke, F. R., Desmet, N., and De Backer, P. (1998). *Excretion studies with 4-androstene-3,17-dione*, Recent advances in doping analysis (6), Proceedings of the Manfred Donike Workshop, 16th Cologne Workshop on Dope Analysis, 15th to 20th March 1998, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke, (Eds,), Verlag Sport und Buch Strauß, Edition Sport, p. 171. Koln, Germany.

Van Eenoo P., F.T. Delbeke, Desmet N. and De Backer P., *Excretion studies with 7-keto-dehydroepiandrosterone*, in Recent Advances in Doping Analysis (9), Proceedings of the 19th Cologne Workshop on Dope Analysis, W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (editors) Sport and Buch Straub, Köln (2001) p. 91

Wright F., J. P. Lafarge, J. Antréassian, M. Lagoguey and G. Péres, in P. Hemmersbach and K. I. Birkerland (eds), *Long term Study of Steroid and Peptidic Hormones in the Plasma of Healthy Young Men under Controlled Testosterone Undecanoate Therapy,* in Blood Sample in Doping Control, Second International Symposium on Drugs in Sports, Lillehammer, Norway (1993), p. 65

IAEA	International Atomic Energy Agency
ISL	International Standard for Laboratories
ISO	International Organization of Standards
IUPAC	International Union of Pure & Applied Chemistry
NBS	National Bureau of Standards
NIST	National Institute of Standards and Technology
RIVM	National Institute of Public Health and the Environment
UCLA	University of California, Los Angeles
VPDB	Vienna PeeDee Belemnite
VSMOW	Vienna Standard Mean Ocean Water

USADA 0847

Application of Gas Chromatography – Combustion – Isotope Ratio Mass Spectrometry to Doping Control

2nd Annual USADA Symposium
on Anti-Doping Science
Los Angeles, California, USA
August 21st – 24th, 2003

Edited By:
Larry D. Bowers, Richard L. Hilderbrand, & Elisha J. Symanski

United States Anti-Doping Agency
Colorado Springs, Colorado, USA

© United States Anti-Doping Agency
2550 Tenderfoot Hill Street, Suite 200
Colorado Springs, CO 80906
Phone 719.785.2000 • Fax 719.785.2001

USADA 0848

NADPH	Nicotinamide-Adenine Dinucleotide Phosphate
NMI	National Measurements Institutes
P2	Pregnanediol
P3	Pregnanetriol
PT	Proficiency Test
PTV	Programmed Temperature Volatilization
RoDH	Retinol DeHydrogenase
RM	Reference material
S	Sulfate
SD	Standard Deviation
SDR	Short-chain dehydrogenase reductase
SI	Systeme International
SPE	Solid Phase Extraction
T	Testosterone
T/E	Ratio of Testosterone/Epi-Testosterone
TBDMS	Tert-Butyl Dimethyl Silyl Group
TC	Target compound
TMIS	Trimethyl iodo Silene
TMP	Total Measurement Process
TMS	Trimethyl Silyl Group
YPD	Yeast extract peptone glucose
VIM	Vocabulary Internationale of Metrology

Miscellaneous	
AGRL	Australian Government Reference Laboratory
AOAC	Association of Analytical Communities
BIPM	International Bureau of Weights & Measures
C-LADA	International Federation of Clinical Chemistry scientific Committee for Laboratory Assessment of Drugs of Abuse

IAEA	International Atomic Energy Agency
ISL	International Standard for Laboratories
ISO	International Organization of Standards
IUPAC	International Union of Pure & Applied Chemistry
NBS	National Bureau of Standards
NIST	National Institute of Standards and Technology
RIVM	National Institute of Public Health and the Environment
UCLA	University of California, Los Angeles
VPDB	Vienna PeeDee Belemnite
VSMOW	Vienna Standard Mean Ocean Water

Application of Gas Chromatography – Combustion – Isotope Ratio Mass Spectrometry to Doping Control

2nd Annual USADA Symposium
on Anti-Doping Science
Los Angeles, California, USA
August 21st – 24th, 2003

Edited By:
Larry D. Bowers, Richard L. Hilderbrand, & Elisha J. Symanski

United States Anti-Doping Agency
Colorado Springs, Colorado, USA

CYP3A4	Cytochrome P450, subfamily IIIA (nifedipine oxidase) polypeptide 4
DHEA	DehydroepiAndrosterone
DHT	Dihydrotestosterone
DTT	Dithiothreitol
E	Etiocholanolone
EA-IRMS	Elemental Analysers - Isotope ratio mass spectrometry
EC	European Commission
Epi-T	Epi-Testosterone
ERC	Endogenous reference compound
G	Glucuronide
GC	Gas chromatography
GC-C-IRMS	Gas chromatography combustion isotope ratio mass spectrometry
GC-MS	Gas chromatography mass spectrometry
hCG	Human Chorionic Gondaotropin
HETP	Heights Equivalent to a Theoretical Plate
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HSD	Hydroxysteroid Dehydrogenases
IM	Intramuscular
IRMS	Isotope ratio mass spectrometry
ISODAT NT	Isotope data systems software
ISOSTER	Project: Determination of the origin of hormones in cattle
LC-MS	Liquid chromatography - mass spectrometry
MS	Mass Spectrometry
MSD	Mass Selective Detector
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
NAD	Nicotinamide-Adenine Dinucleotide

USADA 0849

Preface

The United States Anti-Doping Agency (USADA) was formed in March, 2000 and assumed formal responsibilities for the testing of Olympic, Paralympic, and Pan American athletes; adjudication of doping cases; education; and research in October, 2000. Since its inception, USADA has made a commitment to contribut to the resolution of scientific issues of concern to the global anti-doping community. In reviewing the contributions that USADA might make to the anti-doping movement, there was a clear need for an annual meeting to bring together anti-doping program administrators, laboratory directors, pharmaceutical industry scientists, and academic scientists with expertise in a specific area of interest to the anti-doping community. This meeting could foster improvements in the system and enhance communication and understanding of the issues in doping control for various stakeholders.

USADA's first Annual Symposium on Anti-Doping Science, held in Atlanta, GA in October, 2002, addressed the technology of, testing for, and detection of Oxygen Transport Enhancing Agents and Methods. Eighty-three scientists, doping program administrators, and medical personnel from various international federations (IFs) met and discussed how to eliminate the use of oxygen transport enhancement. As a result of the meeting, critical research needs were identified. These needs have been addressed by both USADA and the World Anti-Doping Agency (WADA) through their respective research grants programs. It is our hope that as a result of this cooperative effort, tests for the oxygen transport enhancing compounds and methods will be part of the routine testing armamentarium of the WADA-accredited laboratories in the near future.

This Second Annual USADA Symposium addressed another difficult issue for anti-doping science – the detection of anabolic steroids that are normally found in the body. The initial approach to detection of abuse of testosterone (T) was the T/epi-T ratio, which has served the anti-doping movement well for a number of years, but which is prone to false negative results. The purpose of this meeting is to discuss the application of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) to Doping Analysis. While great progress has been made, particularly in the past five years, this potentially valuable technique has not been widely implemented. There are several reasons for this, but it is clear that definition of the remaining issues and identification of key areas that

need to be resolved scientifically will play a critical role in wider implementation of the technique.

USADA would like to express its thanks to Dr. Don Catlin, a leader in the use of GC-C-IRMS, and to University of California, Los Angeles (UCLA) for their assistance in the planning of the Symposium.

USADA 0850

Acronym List

Sport Related		
Acronym	**Definition**	**Description**
CAS	Court of Arbitration for Sport	Court established to hear cases involving sport
IF	International Federation	Organization responsible for the worldwide governance of a particular sport
IOC	International Olympic Committee	
NADO	National Anti-Doping Organization	Organization responsible for the drug testing of elite athletes in a particular country
NFL	National Football League	Organization responsible for the sport of football I the U.S.
UCI	Union Cycliste Internationale	International Federation for Cycling
USADA	United States Anti-Doping Agency	Organization responsible for the drug testing of Olympic, Paralympic, and Pan-American elite athletes throughout the United States
WADA	World Anti-Doping Agency	Organization responsible for the drug testing of elite athletes throughout the world

Scientific and Medical	
17βHSD	17 β hydroxysteroid dehydrogenase
3βHSD	3β–hydroxysteroid dehydrogenase
3-PGA	3-Phosphoglyceric Acid
A	Androsterone
Adiol	Androstanediol
AED	Androstenedione
AKR	Aldo-keto reductase
CITAC	Co-Operation on International Traceability in Analytical Chemistry

CYP3A4	Cytochrome P450, subfamily IIIA (nifedipine oxidase) polypeptide 4
DHEA	DehydroepiAndrosterone
DHT	Dihydrotestosterone
DTT	Dithiothreitol
E	Etiocholanolone
EA-IRMS	Elemental Analysers - Isotope ratio mass spectrometry
EC	European Commission
Epi-T	Epi-Testosterone
ERC	Endogenous reference compound
G	Glucuronide
GC	Gas chromatography
GC-C-IRMS	Gas chromatography combustion isotope ratio mass spectrometry
GC-MS	Gas chromatography mass spectrometry
hCG	Human Chorionic Gondaotropin
HETP	Heights Equivalent to a Theoretical Plate
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HSD	Hydroxysteroid Dehydrogenases
IM	Intramuscular
IRMS	Isotope ratio mass spectrometry
ISODAT NT	Isotope data systems software
ISOSTER	Project: Determination of the origin of hormones in cattle
LC-MS	Liquid chromatography - mass spectrometry
MS	Mass Spectrometry
MSD	Mass Selective Detector
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
NAD	Nicotinamide-Adenine Dinucleotide

Preface

The United States Anti-Doping Agency (USADA) was formed in March, 2000 and assumed formal responsibilities for the testing of Olympic, Paralympic, and Pan American athletes; adjudication of doping cases; education; and research in October, 2000. Since its inception, USADA has made a commitment to contribut to the resolution of scientific issues of concern to the global anti-doping community. In reviewing the contributions that USADA might make to the anti-doping movement, there was a clear need for an annual meeting to bring together anti-doping program administrators, laboratory directors, pharmaceutical industry scientists, and academic scientists with expertise in a specific area of interest to the anti-doping community. This meeting could foster improvements in the system and enhance communication and understanding of the issues in doping control for various stakeholders.

USADA's first Annual Symposium on Anti-Doping Science, held in Atlanta, GA in October, 2002, addressed the technology of, testing for, and detection of Oxygen Transport Enhancing Agents and Methods. Eighty-three scientists, doping program administrators, and medical personnel from various international federations (IFs) met and discussed how to eliminate the use of oxygen transport enhancement. As a result of the meeting, critical research needs were identified. These needs have been addressed by both USADA and the World Anti-Doping Agency (WADA) through their respective research grants programs. It is our hope that as a result of this cooperative effort, tests for the oxygen transport enhancing compounds and methods will be part of the routine testing armamentarium of the WADA-accredited laboratories in the near future.

This Second Annual USADA Symposium addressed another difficult issue for anti-doping science – the detection of anabolic steroids that are normally found in the body. The initial approach to detection of abuse of testosterone (T) was the T/epi-T ratio, which has served the anti-doping movement well for a number of years, but which is prone to false negative results. The purpose of this meeting is to discuss the application of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) to Doping Analysis. While great progress has been made, particularly in the past five years, this potentially valuable technique has not been widely implemented. There are several reasons for this, but it is clear that definition of the remaining issues and identification of key areas that

- The Certificate of Analysis or Laboratory Report should include the following
 - Names of target compounds
 - Name(s) of the ERCs
 - $\delta^{13}C$ values of the target compound(s) and the ERC
 - Difference between $\delta^{13}C$ values of the target compound(s) and the ERC
 - Any other information used to determine that the sample was positive (e.g., reference ranges for the individual target compounds)
 - Any opinions should be clearly labeled as such
- Delta values are important and should be in the report
 - Reporting of atom % excess ^{13}C in parts per million may be a more appropriate means of reporting the data. This figure can be derived, for example, from the difference of $\delta^{13}C$ values between the target compound and the ERC.
- The WADA International Standard for Laboratories and Technical Document for Laboratory Documentation Packages apply, although there are specific reporting issues that should be addressed for GC-C-IRMS analysis. The amount of documentation should allow a third party expert to confirm the conclusions derived from the data.
 - Due to the nature of the data, chromatograms should not be required
 - Some measure of peak shape and integrity should be reported
 - Standards for performance verification of the system need to be developed
 - As a system measuring a "quantity," multiple replicates should be analyzed/injected

USADA 0851

Table of Contents

L. Bowers 1
Introduction: Application of Gas Chromatography - Combustion - Isotope Ratio Mass Spectrometry to Doping Control – Defining the Issues

Attendee List 6
Recommendations and Conclusions 12

SESSION 1: BASICS OF GC-C-IRMS IN DOPING CONTROL

R. Auchus 15
Human Anabolic/Androgenic Steroid Synthesis and Metabolism

D. Matthews 22
Fundamentals of compound identification and measurement using isotopes and mass spectrometry

SESSION 2: APPLICATION OF GC-C-IRMS TO DOPING CONTROL

M. Thevis 29
$^{13}C/^{12}C$-Measurement of 17-Ketosteroids to detect Abuse of Androgenic Steroids in Sport

D. Catlin 35
GC-C-IRMS Research at UCLA

J. Segura 42
GC-C-IRMS for endogenous steroids "Challenging the System"

M. Ueki 48
Improved detection of testosterone cascades by monitoring delta-parameters of Androsterone, etiocholanolone, 5 α- and 5 β-androstandiols and DHEA

B. Ahrens 54
Effect of High Soy Diet on GC-C-IRMS Results

B. Le Bizec 58
GC-C-IRMS to Control the Misuse of Natural Occurring Steroid in Breeding Animals

Summary of Discussions on Basics and Application of GC-C-IRMS to Doping Control 63

SESSION 3: UNCERTAINTY IN GC-C-IRMS MEASUREMENT AS APPLIED TO DOPING CONTROL

M. Verkouteren 67
Standardization of Isotope Ratio Measurements for Doping Control

T. Brenna 75
Effects of Chromatographic Overlap on Uncertainty

A. Preiss-Weigert 82
ISOSTER Determination of the Origin of Hormones in Cattle

Summary of Discussions on Uncertainty in GC-C-IRMS Measurement as Applied to Doping Control 86

SESSION 4: HARMONIZATION OF INTER-LABORATORY RESULTS

R. Kazlauskas 89
Some preliminary results on the carbon isotope ratios of ketonic steroids in urines collected from different countries

M. Becchi 94
Towards new $\delta^{13}C$ signature for steroids?

A. Phillips 97
GVI Inter-Laboratory Harmonization

C. Douthitt 101
GC-C-IRMS – getting the right numbers

Summary of Discussions on Harmonization of Inter-Laboratory Results 103
Summary of Discussions on Harmonization of Reporting 105

Acronym List 107

USADA 0852

Summary of Discussions:

Harmonization of Reporting

- The Certificate of Analysis or Laboratory Report should include the following
 - Names of target compounds
 - Name(s) of the ERCs
 - $\delta^{13}C$ values of the target compound(s) and the ERC
 - Difference between $\delta^{13}C$ values of the target compound(s) and the ERC
 - Any other information used to determine that the sample was positive (e.g., reference ranges for the individual target compounds)
 - Any opinions should be clearly labeled as such
- Delta values are important and should be in the report
 - Reporting of atom % excess ^{13}C in parts per million may be a more appropriate means of reporting the data. This figure can be derived, for example, from the difference of $\delta^{13}C$ values between the target compound and the ERC.
- The WADA International Standard for Laboratories and Technical Document for Laboratory Documentation Packages apply, although there are specific reporting issues that should be addressed for GC-C-IRMS analysis. The amount of documentation should allow a third party expert to confirm the conclusions derived from the data.
 - Due to the nature of the data, chromatograms should not be required
 - Some measure of peak shape and integrity should be reported
 - Standards for performance verification of the system need to be developed
 - As a system measuring a "quantity," multiple replicates should be analyzed/injected

Table of Contents

L. Bowers
Introduction: Application of Gas Chromatography - Combustion - Isotope Ratio Mass Spectrometry to Doping Control – Defining the Issues 1

Attendee List 6
Recommendations and Conclusions 12

SESSION 1: BASICS OF GC-C-IRMS IN DOPING CONTROL

R. Auchus
Human Anabolic/Androgenic Steroid Synthesis and Metabolism 15

D. Matthews
Fundamentals of compound identification and measurement using isotopes and mass spectrometry 22

SESSION 2: APPLICATION OF GC-C-IRMS TO DOPING CONTROL

M. Thevis
$^{13}C/^{12}C$-Measurement of 17-Ketosteroids to detect Abuse of Androgenic Steroids in Sport 29

D. Catlin
GC-C-IRMS Research at UCLA 35

J. Segura
GC-C-IRMS for endogenous steroids "Challenging the System" 42

M. Ueki
Improved detection of testosterone cascades by monitoring delta-parameters of Androsterone, etiocholanolone, 5 α- and 5 β-androstandiols and DHEA 48

B. Ahrens
Effect of High Soy Diet on GC-C-IRMS Results 54

B. Le Bizec
GC-C-IRMS to Control the Misuse of Natural Occurring Steroid in Breeding Animals 58

Summary of Discussions on Basics and Application of GC-C-IRMS to Doping Control 63

- Reference materials should be developed and used as internal and external standards in order to evaluate the performance of the system (sample preparation, GC separation, combustion chamber, etc.) and to increase uniformity of between laboratory results
- The use of different target compounds and different ERCs will result in differences between laboratories.
- A WADA-certified batch of characterized acetic anhydride should be made available to all laboratories
- A study comparing **A/E** vs diol test should be completed to assess sensitivity and specificity

USADA 0853

Introduction: Application of Gas Chromatography – Combustion - Isotope Ratio Mass Spectrometry to Doping Control- Defining the Issues

Larry D. Bowers, Ph.D.
Senior Managing Director
USADA

The history of the application of isotopic analysis to doping control began with the 1990 abstract by Southan and his colleagues (1) reporting that there was a difference in ^{13}C content between **T** produced in the gonads and pharmaceutical **T**. Both instrumental and sample preparation limitations slowed the development of a routine method with sufficient sensitivity to detect nanogram quantities of steroid and sufficient specificity to detect small differences in ^{13}C content. Michel Becchi and his colleagues (2) reported a GC-C-IRMS method applicable to urine samples in 1994. In the intervening years, several groups, including those of Michel Becchi (3,4), Cedric Shackleton (5,6), Willi Schänzer (7,8,11), Don Catlin (9,12-14) and others (10,15,16), all reported significant steps forward in the measurement of isotopically-enriched steroids using GC-C-IRMS. From this progress, four approaches to GC-C-IRMS analysis are emerging – those using underivatized steroid versus those using a derivatization of the steroid prior to GC analysis and those measuring Androsterone (**A**) and etiocholanolone (**E**) versus those measuring androstanediols (Adiols). Each of these approaches has advantages and disadvantages, and discussion of these strengths and weaknesses will be a major theme of the meeting. In addition, various biological and physiological issues remain to be resolved. For example, several groups have reported a difference in delta values between 5α-Adiol and 5β-Adiol, but the biochemical explanation of this phenomenon is not clear. Shackleton et al (5) observed that diet plays a significant role in establishing the absolute value of the $^{13}C/^{12}C$ ratio. Finally, harmonization of reporting among the laboratories will be an important step toward wider use of GC-C-IRMS.

Another important step in application of GC-C-IRMS testing is its recognition in the anti-doping rules in sport. In 2000, the anti-doping rules of sport began to mention IRMS. For example, the Federation Internationale de Natation (FINA) rules (17) state:

"In the case of a T/E greater than 6, and **in the absence of an isotope ratio measurement analysis** (*emphasis added*) establishing the presence of exogenous **T**, or its metabolites, or establishing that the **T** analyzed is endogenous, it is mandatory that the relevant medical authority conducts an investigation before the sample is declared positive."

The International Association of Athletics Federations (IAAF) rules (18) state:

"Evidence from ... isotopic ratio measurements may be used to draw **definite conclusions** *(emphasis added)*."

The Union Cycliste Internationale (UCI) has included the following statement in their rules (19):

"The Antidoping Commission can have any sample or part of a sample analyzed by a Gas Chromatography/Combustion/ Isotope Ratio Mass Spectrometry in any laboratory which has this apparatus... **If this analysis shows an exogenous application** (*emphasis added*), the rider will be considered positive. In other cases, the Antidoping Commission can ask for further tests..."

And finally in the 2003 International Olympic Committee (IOC)/WADA Prohibited List Explanatory Notes (20), the following footnote is found:

"6. In prohibited methods, attention is drawn to the fact that Isotope Ratio Mass Spectrometry (IRMS) is considered the preferred method of detecting doping with endogenous androgenic anabolic steroids. It is therefore recommended that IRMS analysis may be applied to any urine that the Laboratory considers has atypical features in the steroid profile."

While this indicates the commitment of these organizations to be on the cutting edge of anti-doping science, the application of GC-C-IRMS to doping control needs thoughtful scientific evaluation.

- Are there doping agents or techniques that could increase **T** concentrations while maintaining a natural $^{13}C/^{12}C$ ratio?
- Are GC-C-IRMS measurements always definitive for an exogenous administration of **T** or its precursors?
- Does GC-C-IRMS replace some of the other analytical tools for doping analysis or does it complement them?

USADA 0854

Summary of Discussions:

Harmonization of Inter-laboratory Results

- Reference materials should be developed and used as internal and external standards in order to evaluate the performance of the system (sample preparation, GC separation, combustion chamber, etc.) and to increase uniformity of between laboratory results
- The use of different target compounds and different ERCs will result in differences between laboratories.
- A WADA-certified batch of characterized acetic anhydride should be made available to all laboratories
- A study comparing A/E vs diol test should be completed to assess sensitivity and specificity

Introduction: Application of Gas Chromatography – Combustion - Isotope Ratio Mass Spectrometry to Doping Control- Defining the Issues

Larry D. Bowers, Ph.D.
Senior Managing Director
USADA

The history of the application of isotopic analysis to doping control began with the 1990 abstract by Southan and his colleagues (1) reporting that there was a difference in ^{13}C content between **T** produced in the gonads and pharmaceutical **T**. Both instrumental and sample preparation limitations slowed the development of a routine method with sufficient sensitivity to detect nanogram quantities of steroid and sufficient specificity to detect small differences in ^{13}C content. Michel Becchi and his colleagues (2) reported a GC-C-IRMS method applicable to urine samples in 1994. In the intervening years, several groups, including those of Michel Becchi (3,4), Cedric Shackleton (5,6), Willi Schänzer (7,8,11), Don Catlin (9,12-14) and others (10,15,16), all reported significant steps forward in the measurement of isotopically-enriched steroids using GC-C-IRMS. From this progress, four approaches to GC-C-IRMS analysis are emerging – those using underivatized steroid versus those using a derivatization of the steroid prior to GC analysis and those measuring Androsterone (**A**) and etiocholanolone (**E**) versus those measuring androstanediols (Adiols). Each of these approaches has advantages and disadvantages, and discussion of these strengths and weaknesses will be a major theme of the meeting. In addition, various biological and physiological issues remain to be resolved. For example, several groups have reported a difference in delta values between 5α-Adiol and 5β-Adiol, but the biochemical explanation of this phenomenon is not clear. Shackleton et al (5) observed that diet plays a significant role in establishing the absolute value of the $^{13}C/^{12}C$ ratio. Finally, harmonization of reporting among the laboratories will be an important step toward wider use of GC-C-IRMS.

Another important step in application of GC-C-IRMS testing is its recognition in the anti-doping rules in sport. In 2000, the anti-doping rules of sport began to mention IRMS. For example, the Federation Internationale de Natation (FINA) rules (17) state:

7. Assuming that points 1-6 are properly addressed, getting the same $\delta^{13}C$ (and δD) in different labs requires very close attention to the (often arcane) details of reference gases, primary and secondary standards. This, however, is not the purview of the manufacturer.

REFERENCES

1. Assonov S.S. and C.A.M. Brenninkmeijer, 2003, **On the ^{17}O correction for CO_2 mass spectrometer isotopic analysis.** *Rapid Communications in Mass Spectrometry*, 17, 1007-1016

USADA 0855

The rules of sport have other important definitions that impact doping control. First, an adverse analytical finding in the WADA Code requires only the detection of a prohibited substance or its metabolites or markers in the sample from the athlete. Second, the principle of strict liability applies meaning that the athlete is held accountable for whatever is found in his or her body or body fluids. It does not matter what the intent was. It does not matter how it got there. Third, the athlete's rights include the right to a hearing before an arbitration panel, such as the Court of Arbitration for Sport (CAS). Finally, the burden of proof for the prosecution in such an arbitration hearing according to the WADA Code is "the comfortable satisfaction of the hearing body bearing in mind the seriousness of the allegation which is being made." This is higher than a preponderance of evidence (as would be in effect in a US civil proceeding) but lower than beyond a reasonable doubt (as in a criminal proceeding). As we discuss the technique of GC-C-IRMS, our focus should be on the scientific reliability of the GC-C-IRMS technique in establishing, or ruling out, the administration of a prohibited substance.

REFERENCES

1. Southan G, Mallet A, Jumeau J, Craig S, Poojara N, et al. Programme and Abstracts of the 2nd International Symposium of Applied Mass Spectrometry in the Health Sciences, Barcelona, Spain, 1990, p 306.
2. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of T in sport. *Rapid Commun Mass Spectrom* 1994; **8**: 304-8.
3. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, et al. Improved method of detection of T abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J Mass Spectrom* 1996; **21**: 169-76.
4. Aguilera R, Becchi M, Grenot C, Casabianca H, Hatton CK. Detection of T misuse: comparison of two chromatographic sample preparation methods for gas chromatographic-combustion/isotope ratio mass spectrometric analysis. *J Chromatogr B Biomed Appl* 1996; **687**:43-53.
5. Shackleton CH, Phillips A, Chang T, Li Y. Confirming T administration by isotope ratio mass spectrometric analysis of urinary Adiols. *Steroids* 1997; **62**: 379-87.

6. Shackleton CH, Roitman E, Phillips A, Chang T. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epi-testosterone, and dehydroepiAndrosterone: potential use in gas chromatography isotope ratio mass spectrometry. *Steroids* 1997; **62**: 665-73.
7. Horning S, Geyer H, Machnik M, Schänzer W, Hilkert A, and Oeßelmann J. Detection of exogenous steroids by $^{13}C/^{12}C$ analysis. In *Recent Advances in Doping Analysis (4) – Proceedings of the 14th Cologne Workshop on Dope Analysis 1996*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 1997. pp 275-83.
8. Horning S, Geyer H, Gotzmann A, Schänzer W. Detection of exogenous steroids by $^{13}C/^{12}C$ analysis. In *Recent Advances in Doping Analysis (5) – Proceedings of the 15th Cologne Workshop on Dope Analysis 1997*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 1998. pp 135-48.
9. Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, et al. Screening urine for exogenous **T** by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. *J Chromatogr B Biomed Sci Appl* 1999; **727**: 95-105.
10. Ueki M, Okano M. Analysis of exogenous dehydroepiAndrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 1999; **13**: 2237-43.
11. Flenker U, Horning S, Nolteernsting E, Geyer H, Schänzer W. Measurement of $^{12}C/^{13}C$-ratios to Confirm Misuse of Endogenous Steroids. In *Recent Advances in Doping Analysis (6) – Proceedings of the 16th Cologne Workshop on Dope Analysis 1998*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 1999. pp 243-56.
12. Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary **A** and **E** $\delta^{13}C$ (‰) values by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2000; **14**: 2294-9.
13. Bourgogne E, Herrou V, Mathurin J-C, Becchi M, de Ceaurriz J. Detection of exogenous intake of natural corticosteroids by gas chromatography/combustion/isotope ratio mass spectrometry : application to misuse in sport. Rapid Commun Mass Spectrom 2000; **14**: 2343-7.

USADA 0856

GC-C-IRMS: getting the right numbers

Charles Douthitt
ThermoFinnigan

The isotope ratio mass spectrometer (IRMS) is designed to precisely and accurately measure the difference in isotopic compositions between a sample and a reference gas. Considerations significant to achieving this goal in the practice of GC-IRMS include:

CAPILLARY GC: *the best possible chromatographic resolution is imperative*

1. Compounds undergo isotope fractionation in a capillary GC column, so that the front of a peak is generally enriched in ^{13}C relative to the tail. It is not possible to perform peak deconvolution on GC-IRMS data, so the most accurate data requires the best possible separation of peaks
2. In order to ensure the most consistent GC performance, the new ThermoFinnigan ISODAT NT software includes full control over GC methods, injectors and auto samplers

GC INTERFACE: *chromatographic resolution can only get worse, it can't be improved*

3. The major design goal for the interface between the capillary GC and the IRMS was to do nothing that could degrade chromatographic resolution (e.g., no cold spots, no T connections, the reactors are small bore and are not packed)

SOFTWARE: *data collection and data correction*

4. The software must properly and consistently perform determination of baselines, peak integration and time shift corrections (required because of the isotope fractionation discussed in #1). The new ISODAT NT software includes significant enhancements to peak integration and baseline correction algorithms
5. Proper correction for the contribution of $^{12}C^{17}O^{16}O$ to mass 45. The new ISODAT NT software incorporates the newest and most powerful methods for performing this correction[1].

IRMS PERFORMANCE: *linearity*

6. While sensitivity is an important consideration (how small a sample can one analyze?), an often overlooked but equally important consideration is linearity. Linearity should be a specification, and it should be small and consistent and it should be possible to correct raw data to eliminate any effect of varying sample size

STANDARDIZATION: *getting onto the V-PDB scale*

7. Assuming that points 1-6 are properly addressed, getting the same $\delta^{13}C$ (and δD) in different labs requires very close attention to the (often arcane) details of reference gases, primary and secondary standards. This, however, is not the purview of the manufacturer.

REFERENCES

1. Assonov S.S. and C.A.M. Brenninkmeijer, 2003, **On the ^{17}O correction for CO_2 mass spectrometer isotopic analysis.** *Rapid Communications in Mass Spectrometry*, 17, 1007-1016

The rules of sport have other important definitions that impact doping control. First, an adverse analytical finding in the WADA Code requires only the detection of a prohibited substance or its metabolites or markers in the sample from the athlete. Second, the principle of strict liability applies meaning that the athlete is held accountable for whatever is found in his or her body or body fluids. It does not matter what the intent was. It does not matter how it got there. Third, the athlete's rights include the right to a hearing before an arbitration panel, such as the Court of Arbitration for Sport (CAS). Finally, the burden of proof for the prosecution in such an arbitration hearing according to the WADA Code is "the comfortable satisfaction of the hearing body bearing in mind the seriousness of the allegation which is being made." This is higher than a preponderance of evidence (as would be in effect in a US civil proceeding) but lower than beyond a reasonable doubt (as in a criminal proceeding). As we discuss the technique of GC-C-IRMS, our focus should be on the scientific reliability of the GC-C-IRMS technique in establishing, or ruling out, the administration of a prohibited substance.

REFERENCES

1. Southan G, Mallet A, Jumeau J, Craig S, Poojara N, et al. Programme and Abstracts of the 2nd International Symposium of Applied Mass Spectrometry in the Health Sciences, Barcelona, Spain, 1990, p 306.
2. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of **T** in sport. *Rapid Commun Mass Spectrom* 1994; **8**: 304-8.
3. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, et al. Improved method of detection of **T** abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J Mass Spectrom* 1996; **21**: 169-76.
4. Aguilera R, Becchi M, Grenot C, Casabianca H, Hatton CK. Detection of **T** misuse: comparison of two chromatographic sample preparation methods for gas chromatographic-combustion/isotope ratio mass spectrometric analysis. *J Chromatogr B Biomed Appl* 1996; **687**:43-53.
5. Shackleton CH, Phillips A, Chang T, Li Y. Confirming **T** administration by isotope ratio mass spectrometric analysis of urinary Adiols. *Steroids* 1997; **62**: 379-87.

AGUILERA
How much difference is there in between one pulse and another one, in delta values?

PHILLIPS
It depends on what the matrix background is that you drop it in on. But the system will still do background subtraction on the reference peak (prior to the reference peak).

USADA 0857

14. Aguilera R, Chapman TE, Starcevic B, Hatton CK, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with **T** based on urine diols: controls and athletes with elevated **T**/epi-T ratios. *Clin Chem* 2001; **47**: 292-300.
15. Aguilera R, Hatton CK, Catlin DH. Detection of epi-T doping by isotope ratio mass spectrometry. *Clin Chem* 2002; **48**: 629-36.
16. Ayotte C, Goudreault D, Lajeunesse A, Cléroux M, Richard Y, et al. GC-C-IRMS and GC/MS in "Natural" Steroids Testing. In *Recent Advances in Doping Analysis (9) – Proceedings of the 19th Cologne Workshop on Dope Analysis 2001*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 2002. pp 133-43.
17. Addendum to FINA Handbook 2002-2005 (December 2002). Appendix A. Section I.C.1., p. 18
18. IAAF Procedural Guidelines for Doping Control, 2000 edition. Schedule 1, Part I(a)(I), p 18.
19. UCI Prohibited Classes of Substances and Prohibited Methods (1st April 2000), Section I.C., p 3.
20. IOC/WADA List of Prohibited Substances

Attendee List

Dr. Rodrigo Aguilera
Researcher
Pasarow Mass Spectrometry Laboratory, UCLA
United States

Mr. Brian Ahrens
Researcher & Certifying Scientist
UCLA Olympic Analytical Laboratory
United States

Dr. Tongtavuch Anukarahanonta
Head of the IOC/WADA Laboratory
National Doping Control Centre, Mahidol University
Thailand

Prof. Francisco Radler Aquino Neto
Head of the IOC/WADA Laboratory
LABDOP-Ladetec/IQ-UFRJ, Centro de Technologia
Brazil

Dr. Richard Auchus
Assistant Professor of Endocrinology and Metabolism
University of Texas
United States

Mr. Michel Becchi
Research Scientist
Centre National de la Recherche Scientifique
France

Mr. William Bock
USADA Counsel
KROGER, GARDIS & REGAS, L.L.P.
United States

Ms. Kate Borg
Board of Directors
USADA
United States

Prof. Dr. Francesco Botré
Head of the IOC/WADA Laboratory
Federazione Medico Sportiva Italiana, Laboratorio Antidoping
Italy

USADA 0858

Derivatized samples have shown to improve the chromatographic separation, but the choice of materials is reduced due to the combustion of the agent attacking the furnace catalyst, also they add extra Carbon atoms that will affect the delta value. Corrections can be made for the addition of the extra Carbons.

To improve the harmonization of the laboratories they need to be analyzing the same test mixture and obtaining the correct values before analyzing the samples. So a Test Mixture needs to be standardized and available to the laboratories.

At present they report the measured delta value of the steroid metabolites, it might be more accurate reporting a ratio values between the different naturally occurring metabolites, and this could eliminate differences between integration logarithms, and also could eliminate the need to correct for the extra carbons added during derivatization.
As long as all the laboratories follow the same protocol then all results should be traceable. Occasional round robin analysis of sets of samples will monitor the laboratories measurements.

Question & Answer Discussion

PHILLIPS
The main thing being that you make sure you put it in the right compound in a position, so it is not going to co-elute with something. I think Prof. Ueki has been adding internal standards with quite a lot of his samples. So straight away, as well as, running standards before you start any samples, you can look down your results and make sure that all your internal standards are staying at the same value.

AGUILERA
You are using the injection of the CO_2 peak still in "backflush off" or "backflush on" in the new software to do the calculation of the value? You better remember if you inject a CO_2 during the "backflush off" and after, you switch to "backflush on". Are you doing the analysis and after you inject again to CO_2 and "backflush off"?

PHILLIPS
That is the standard way we would do analysis but you can actually put the CO_2 pulse the same time that the GC flow is going in.

AGUILERA
How much difference is there in between one pulse and another one, in delta values?

PHILLIPS
It depends on what the matrix background is that you drop it in on. But the system will still do background subtraction on the reference peak (prior to the reference peak).

14. Aguilera R, Chapman TE, Starcevic B, Hatton CK, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with T based on urine diols: controls and athletes with elevated T/epi-T ratios. *Clin Chem* 2001; **47**: 292-300.
15. Aguilera R, Hatton CK, Catlin DH. Detection of epi-T doping by isotope ratio mass spectrometry. *Clin Chem* 2002; **48**: 629-36.
16. Ayotte C, Goudreault D, Lajeunesse A, Cléroux M, Richard Y, et al. GC-C-IRMS and GC/MS in "Natural" Steroids Testing. In *Recent Advances in Doping Analysis (9) – Proceedings of the 19th Cologne Workshop on Dope Analysis 2001*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 2002. pp 133-43.
17. Addendum to FINA Handbook 2002-2005 (December 2002). Appendix A. Section I.C.1., p. 18
18. IAAF Procedural Guidelines for Doping Control, 2000 edition. Schedule 1, Part I(a)(I), p 18.
19. UCI Prohibited Classes of Substances and Prohibited Methods (1st April 2000), Section I.C., p 3.
20. IOC/WADA List of Prohibited Substances

The original developments for stable isotopes techniques were mostly dedicated to geochemistry applications. With the evolution of the techniques the scientists working in the field of Cosmo chemistry, hydrology, environment, doping control, food control (adulteration), medical research, pharmaceutical industry and now in forensic science as well have adopted stable isotope analysis as an essential tool in their laboratories.

Misuse of T has become a classical doping case in sport. The IOC and all sport IFs ban its use. Considering the natural variability of T concentration in individuals coming from different origins, measuring concentrations of T metabolites in urines was not specific enough and a method to differentiate natural and synthetic T was needed, so GC IRMS was introduced.

At present sports laboratories are analyzing steroid metabolites by GC IRMS and compiling huge libraries of results on gender, origin and diets, but the questions lies can the same sample give the same result if measured in another laboratory on a different instrument?

The answer to this question is yes.

However the laboratories will have to harmonize their approach to the analysis. This means that every analysis has to be traceable to the Instrument calibration material, which in turn must be traceable to the international standard VPDB for CO2 measurements.

At present there are many commercial standards traceable to VPDB, that can be obtained from NIST, EIL and IAEA but none of them can be analyzed by GC. This leaves some laboratories that do not have other isotope techniques, vulnerable at not being able to manufacture calibrated secondary standards. It is important that certain criteria are achievable to give the correct values before attempting to measure any sample by GC IRMS. They are combustion, water removal, sample peak integration and the reference gas that has been calibrated with respect to an international standard or against the secondary standards. The correct delta values of the GC test material must be obtained before analyzing any samples, the test material should be analyzed at frequent intervals between sample analyses.

Sample preparation is extremely important and the cleaner the sample then the more accurate and reproducible the results will be. Unlike other forms of GC IMS, IRMS does not have high resolution or MS/MS capabilities in being selective. Any impurities around the sample peaks that enter the combustion furnace will be combusted to CO_2 and enter the IRMS.

USADA 0859

Dr. Larry Bowers
Senior Managing Director
USADA
United States

Prof. James Thomas Brenna
Professor
Cornell University
United States

Mr. Stephane Carando
Supervisor in IOC/WADA Laboratory
National Doping Laboratory, CREPS
France

Prof. Dr. Don Catlin
Head of the IOC/WADA Laboratory
UCLA Olympic Analytical Laboratory
United States

Dr. Richard Cohen
Board of Directors
USADA
United States

Mrs. Carol Congdon
Executive Administrator
USADA
United States

Prof. David Cowan
Head of the IOC/WADA Laboratory
Drug Control Centre, King's College London
United Kingdom

Prof. Dr. FT. Frans Delbeke
Head of the IOC/WADA Laboratory
Doping Control Laboratory, Ghent University
Belgium

Dr. Bryan Finkle
Consultant, Forensic Toxicologist
National Football League (NFL)
Unites States

Dr. Jean Fourcroy
Board of Directors
USADA
United States

Dr. Mats Garle
Head of the IOC/WADA Laboratory
Doping Control Laboratory, Huddinge University Hospital
Sweden

Dr. Costas Georgakopoulos
Head of the IOC/WADA Laboratory
Olympic Athletics Center of Athens
Greece

Mr. Joachim Grosse
German Sports University
Germany

Dr. Ralph Hale
Board of Directors
USADA
United States

Dr. Caroline Hatton
Consultant
UCLA Olympic Analytical Laboratory
United States

Prof. Peter Hemmersbach
Head of the IOC/WADA Laboratory
Hormone Laboratory, Aker University Hospital
Norway

Dr. Richard Hilderbrand
Science Director
USADA
United States

Prof. Edward Houghton
Chief Scientist, Director
Horseracing Forensic Laboratory
United Kingdom

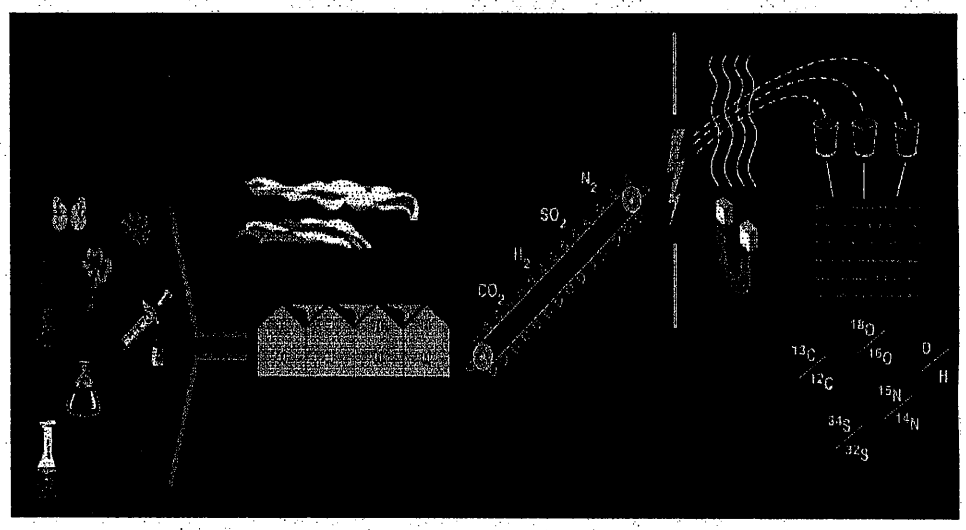
Dr. Marilyn Huestis
Acting Chief, Chemistry and Drug Metabolism Section
National Institute on Drug Abuse
United States

USADA 0860

Inter Laboratory Harmonization

Andy Phillips
GV Instruments

The power of stable isotopes techniques lies in the fact that Isotope Ratio Mass Spectrometry (IRMS) can provide information relating to the origin of the analyzed compounds. That can be used to differentiate natural/artificial compounds, endogenous/exogenous compounds, to determine metabolic pathways, geographic origins…The stable isotope mass spectrometer is a gas source instrument which can analyze very precisely very small variations on the isotopic composition of a limited number of gas species. Typically, $^{13}C/^{12}C$ is measured as CO_2, $^{15}N/^{14}N$ is measured as N_2, D/H is measured as H_2, $^{18}O/^{16}O$ can be measured as CO_2, CO or O_2; $^{34}S/^{32}S$ is measured as SO_2 or SO. Although in the past, the key to stable isotope determinations was the performance of the mass spectrometer itself, today an essential part of any IRMS system is the inlet system that will automatically transform the sample into gas species, which the IRMS can analyze. Stable isotopes can be used at natural abundance level, looking at the natural variations of isotope ratios during biogeochemical processes, or they can be used with tracer compounds artificially enriched in one of the isotopes.



The original developments for stable isotopes techniques were mostly dedicated to geochemistry applications. With the evolution of the techniques the scientists working in the field of Cosmo chemistry, hydrology, environment, doping control, food control (adulteration), medical research, pharmaceutical industry and now in forensic science as well have adopted stable isotope analysis as an essential tool in their laboratories.

Misuse of T has become a classical doping case in sport. The IOC and all sport IFs ban its use. Considering the natural variability of T concentration in individuals coming from different origins, measuring concentrations of T metabolites in urines was not specific enough and a method to differentiate natural and synthetic T was needed, so GC IRMS was introduced.

At present sports laboratories are analyzing steroid metabolites by GC IRMS and compiling huge libraries of results on gender, origin and diets, but the questions lies can the same sample give the same result if measured in another laboratory on a different instrument?

The answer to this question is yes.

However the laboratories will have to harmonize their approach to the analysis. This means that every analysis has to be traceable to the Instrument calibration material, which in turn must be traceable to the international standard VPDB for CO2 measurements.

At present there are many commercial standards traceable to VPDB, that can be obtained from NIST, EIL and IAEA but none of them can be analyzed by GC. This leaves some laboratories that do not have other isotope techniques, vulnerable at not being able to manufacture calibrated secondary standards. It is important that certain criteria are achievable to give the correct values before attempting to measure any sample by GC IRMS. They are combustion, water removal, sample peak integration and the reference gas that has been calibrated with respect to an international standard or against the secondary standards. The correct delta values of the GC test material must be obtained before analyzing any samples, the test material should be analyzed at frequent intervals between sample analyses.

Sample preparation is extremely important and the cleaner the sample then the more accurate and reproducible the results will be. Unlike other forms of GC IMS, IRMS does not have high resolution or MS/MS capabilities in being selective. Any impurities around the sample peaks that enter the combustion furnace will be combusted to CO_2 and enter the IRMS.

Dr. Larry Bowers
Senior Managing Director
USADA
United States

Prof. James Thomas Brenna
Professor
Cornell University
United States

Mr. Stephane Carando
Supervisor in IOC/WADA Laboratory
National Doping Laboratory, CREPS
France

Prof. Dr. Don Catlin
Head of the IOC/WADA Laboratory
UCLA Olympic Analytical Laboratory
United States

Dr. Richard Cohen
Board of Directors
USADA
United States

Mrs. Carol Congdon
Executive Administrator
USADA
United States

Prof. David Cowan
Head of the IOC/WADA Laboratory
Drug Control Centre, King's College London
United Kingdom

Prof. Dr. FT. Frans Delbeke
Head of the IOC/WADA Laboratory
Doping Control Laboratory, Ghent University
Belgium

Dr. Bryan Finkle
Consultant, Forensic Toxicologist
National Football League (NFL)
Unites States

Dr. Jean Fourcroy
Board of Directors
USADA
United States

my lab. Yes, you have to get the strain though, and my understanding is the industrial partner, like you said, decided this was not going to be economically feasible. So, those strains are probably frozen away somewhere and are probably not going to be used for production of hydrocortisone. To answer your question about, can you make androgens? We make DHEA with yeast all the time, just adding cytochrome B5 to the cocktail they have there, to increase the lyase activity. So, it would be a trivial step to do that.

The difference in the delta values is quite amazing. Do you know if those delta values are also different if you take regular yeast and isolate the ergosterol, their normal product, which could potentially be a source of synthetic androgens from the standard YPD cocktail, what you would get?

BECCHI
Yes, the value would be totally very different but have not measured the ergosterol, for instance. It is possible to do this.

USADA 0861

Dr. Rymantas Kazlauskas
Head of the IOC/WADA Laboratory
Australian Government Analytical Laboraories
Australia

Dr. Aishah Latiff
Head of the IOC/WADA Laboratory
Doping Control Centre, Universiti Sains
Malaysia

Prof. Bruno Le Bizec
Head Deputy - Scientific Manager
LABERCA - French Reference Laboratory
France

Mr. Antti Leinonen
Technical Manager in IOC/WADA Laboratory
Doping Control Laboratory, United Laboratories Ltd
Finland

Mrs. Hanne Solveig Lund
Scientist
Hormone Laboratory, Aker University Hospital
Norway

Mr. Terry Madden
Chief Executive Officer
USADA
United States

Prof. Dwight Matthews
Professor & Chair, Chemistry & Medicine
The University of Vermont
United States

Mr. Andy Phillips
Demonstration Laboratory Manager
GV Instruments
United Kingdom

Dr. Angelika Preiss-Weigert
Deputy Head of Instrumental Analysis
Federal Institute for Risk Assessment
Germany

Dr. Doug Rollins
Professor, Pharmacology and Toxicology
Center for Human Toxicology, University of Utah
United States

Mr. Edward Ryan
Sports Medicine Director
U.S. Olympic Committee
United States

Dr. Martial-Olivier Saugy
Head of the IOC/WADA Laboratory
Laboratory for doping analyses, Instiut Universitaire de Medecine Legale
Switzerland

Prof. Jordi Segura
Head of the IOC/WADA Laboratory
Institut Municipal Investigació Mèdica
Spain

Mr. Michael Sekera
Scientific Director
UCLA Olympic Analytical Laboratory
United States

Dr. Matthew Slawson
Assistant Director
Center for Human Toxicology, University of Utah
United States

Mrs. Saskia Sterk
Head Section Veterinary Drugs
National Institute of Public Health and the Environment (RIVM)
Netherlands

Dr. Hiraku Suzuki
Chief Manager/Special Chemistry Group
Mitsubishi Kagaku Bio-Clinical Laboratories INC.
Japan

Mrs. Elisha Symanski
Operations Manager
USADA
United States

Dr. Mario Thevis
German Sports University
Germany

Mr. Travis Tygart
Legal Affairs Director
USADA
Unites States

USADA 0862

C-IRMS differentiation must be done with attention as this corticosteroid metabolite can have an exogenous origin.

REFERENCES

1. *Self-sufficient biosynthesis of pregnenolone and progesterone in engineered yeast*, C. Duport, R. Spagnoli, E. Degryse, and D. Pompon - Nature Biotechnology 16 (1998), 186-189.
2. *Total biosynthesis of hydrocortisone from a simple carbon source in yeast*, B. Dumas *et al.*-Nature Biotechnology 21 (2003), 143-149.
3. *Detection of exogenous intake of natural corticosteroids by GC-C-IRMS: application to misuse in sport*, E. Bourgogne, V. Herrou, J.-C. Mathurin, M. Becchi and J. de Ceaurriz – Rapid Communications in Mass Spectrometry 14 (2000) 233-2347.

Question & Answer Discussion

BECCHI
I think at the moment it is an economical program, the moment the steroids from plants more economical than the yeast, and they are maybe to improve their methods. Actually in the publication, the rate of production was 10 mcg per ml of culture, so it will depend on the economical programs condition.

BOWERS
Do you have plans to analyze the corticosteroid products from yeast?

BECCHI
No, I am no longer involved in this field.

VERKOUTEREN
I was just interested in this designer yeast too. Can the yeast replicate itself and maintain the same functionality?

BECCHI
I do not know at the moment. You have to ask Dr. Pompons who is the specialist on the yeast.

AUCHUS
I know I use Dr. Pompons' genetically altered yeast, not this strain, but we use his system for expressing some of our enzymes in yeast all the time in

my lab. Yes, you have to get the strain though, and my understanding is the industrial partner, like you said, decided this was not going to be economically feasible. So, those strains are probably frozen away somewhere and are probably not going to be used for production of hydrocortisone. To answer your question about, can you make androgens? We make DHEA with yeast all the time, just adding cytochrome B5 to the cocktail they have there, to increase the lyase activity. So, it would be a trivial step to do that.

The difference in the delta values is quite amazing. Do you know if those delta values are also different if you take regular yeast and isolate the ergosterol, their normal product, which could potentially be a source of synthetic androgens from the standard YPD cocktail, what you would get?

BECCHI
Yes, the value would be totally very different but have not measured the ergosterol, for instance. It is possible to do this.

Dr. Rymantas Kazlauskas
Head of the IOC/WADA Laboratory
Australian Government Analytical Laboraories
Australia

Dr. Aishah Latiff
Head of the IOC/WADA Laboratory
Doping Control Centre, Universiti Sains
Malaysia

Prof. Bruno Le Bizec
Head Deputy - Scientific Manager
LABERCA - French Reference Laboratory
France

Mr. Antti Leinonen
Technical Manager in IOC/WADA Laboratory
Doping Control Laboratory, United Laboratories Ltd
Finland

Mrs. Hanne Solveig Lund
Scientist
Hormone Laboratory, Aker University Hospital
Norway

Mr. Terry Madden
Chief Executive Officer
USADA
United States

Prof. Dwight Matthews
Professor & Chair, Chemistry & Medicine
The University of Vermont
United States

Mr. Andy Phillips
Demonstration Laboratory Manager
GV Instruments
United Kingdom

Dr. Angelika Preiss-Weigert
Deputy Head of Instrumental Analysis
Federal Institute for Risk Assessment
Germany

Dr. Doug Rollins
Professor, Pharmacology and Toxicology
Center for Human Toxicology, University of Utah
United States

Towards new $\delta^{13}C$ signature for steroids?

Michel Becchi
Centre National de la Recherche Scientifique

Gonadal **T** is made from precursor molecules derived from a wide variety of food materials. In human bodies, the ^{13}C content of **T** and its metabolites are generally found at about -25‰ for $\delta\ ^{13}C$ values, depending on all the nature of the food eaten and the discrimination of the enzymes involve in the biosynthesis pathways. Synthetic **T** is made from a plant species, mostly soy, with $\delta\ ^{13}C$ values of about -29‰, allowing differentiation of the human one.

The production of pregnenolone, an important intermediate of steroidal drug synthesis was reported from a simple carbon source (ethanol) by recombinant yeast *Saccharomyces cerevisiae* strains (1).

We studied the influence of carbon source on the resulting pregnenolone production from this engineered yeast. The carbon source came from ethanol with two different ^{13}C values (ethanol from sugar cane and from sugar beet). The results of $\delta\ ^{13}C$ determination were presented in the following Table:

Compounds	$^{13}C\ \delta$‰
Pregnenolone acetate from SIGMA Carbon source: plant sterols?	- 36.1
Pregnenolone acetate from Yeast Carbon source : Ethanol from sugar beet	- 19.0
Ethanol from sugar beet	- 26.5
Pregnenolone acetate from Yeast Carbon source : Ethanol from sugar cane	- 3.1
Ethanol from sugar cane	- 12.1

There was a strong discrimination of carbon incorporation into the biosynthetic recombinant yeast steroids. The $\delta\ ^{13}C$ was very different from the natural human steroids one.

More recently, hydrocortisone and cortisone were also produced by other recombinant yeast (2). Corticosteroids could easily be studied by GC-C-IRMS as androstan-tri-one derivatives (3), and, as anabolic steroids, the differentiation between synthetic and natural human ones was effective. The choice of 11-hydroxy-etiocholanolone as an ERCs for anabolic steroids GC-

USADA 0863

Dr. Makoto Ueki
Head of the IOC/WADA Laboratory
Mitsubishi Kagaku Bio-Clinical Laboratories, Inc.
Japan

Dr. Michael Verkouteren
Research Chemist
National Institute of Standards and Technology
United States

Prof. Christina Wang
Program Director
Harbor-UCLA Medical Center
United States

Mr. Rich Wanninger
Communications & Public Affairs Director
USADA
United States

Ms. Andrea Wickerham
Legal Relations and Policy Director
The National Center for Drug Free Sport
United States

Dr. Prof. Mountian Wu
Head of the IOC/WADA Laboratory
China Doping Control Center
China

Mr. Richard Young
USADA Counsel
Holme, Roberts & Owen, LLP
United States

Dr. Mario Zorzoli
Official Doctor
Union Cycling Internationale (UCI)
Switzerland

Recommendations and Conclusions

- A small expert panel should be convened to consider what specific assay improvements are needed, guidelines should be used, and make recommendations to WADA. These recommendations may include guidelines on assay characteristics and performance criteria that will unify the method(s) that are used in doping control.
 - Build on the foundation provided by the IOC Working Group
 - Develop a Proficiency Testing Round Robin to improve harmonization of reported values

- USADA should fund a 50-subject reference range study in the eleven labs currently reporting GC-C-IRMS data
 - Reference material and/or internal standard must be identified and produced
 - Target substances must be identified and run in all samples
 - Specify internal standard and reporting requirements

- There are ways to beat the GC-C-IRMS test, such as administration of hCG, aromatase inhibitors, or clomiphene.
 - Doping may have occurred, even in the presence of a negative GC-C-IRMS test;
 - A positive GC-C-IRMS does definitively support the administration of pharmaceutical **T** or **T** precursors
 - An expert panel needs to provide recommendations on the combination of longitudinal testing and GC-C-IRMS to provide cost-effective testing strategies
 - A GC-C-IRMS may be required in every case of an elevated T/E ratio

- The reporting of differences of $\delta^{13}C$ values (using an endogenous reference compound) as opposed to the ratio of $\delta^{13}C$ values is strongly recommended. There may be additional benefit in considering the absolute $\delta^{13}C$ values compared to the reference ranges of individual target analytes.

- A Reference Material and/or Internal Standard material should be developed to assist the laboratories in achieving more uniform results.

USADA 0864

are going to get a lot of false negatives, unless we can get the criteria really well worked out.

COWEN
Have you considered how the approach you are using is going to fit in to International Standard for Laboratories (ISL) of WADA for next year? Dr. Bowers' comments about screening, confirmatory test. How are you going to apply it? Might the ISL need a separate document to deal with GC-C-IRMS? I was thinking about some discrimination between the screening method, and the confirmatory method that we are required to do.

KAZLAUSKAS
If you are using this as a screening and you actually insist the confirmation be something completely different, in which we have to say the diols, rather than be able to repeat this, or have some other procedure. I do not know.

AGUILERA
Do you know why in some cases the distribution is very tight and some populations it is spread out? I do not think about the shift in the average but talking about the distribution for in the same cases you going from –15 to –25 and then other cases you are using a very tight distribution of the population.

KAZLAUSKAS
Yes, I can think of lots of reasons why they might. I mean maybe these athletes travel. A lot of it is diet related and it may be they travel. They may be mixed populations in terms of genetics. We know for example in the T/E, the Asian population is mixed. You have got those who have got very low T/Es, and you got those who have a T/E like Caucasians.

Towards new $\delta^{13}C$ signature for steroids?

Michel Becchi
Centre National de la Recherche Scientifique

Gonadal T is made from precursor molecules derived from a wide variety of food materials. In human bodies, the ^{13}C content of T and its metabolites are generally found at about -25‰ for $\delta\ ^{13}C$ values, depending on all the nature of the food eaten and the discrimination of the enzymes involve in the biosynthesis pathways. Synthetic T is made from a plant species, mostly soy, with $\delta\ ^{13}C$ values of about -29‰, allowing differentiation of the human one.

The production of pregnenolone, an important intermediate of steroidal drug synthesis was reported from a simple carbon source (ethanol) by recombinant yeast *Saccharomyces cerevisiae* strains (1).

We studied the influence of carbon source on the resulting pregnenolone production from this engineered yeast. The carbon source came from ethanol with two different ^{13}C values (ethanol from sugar cane and from sugar beet). The results of $\delta\ ^{13}C$ determination were presented in the following Table:

Compounds	^{13}C δ‰
Pregnenolone acetate from SIGMA Carbon source: plant sterols?	- 36.1
Pregnenolone acetate from Yeast Carbon source : Ethanol from sugar beet	- 19.0
Ethanol from sugar beet	- 26.5
Pregnenolone acetate from Yeast Carbon source : Ethanol from sugar cane	- 3.1
Ethanol from sugar cane	- 12.1

There was a strong discrimination of carbon incorporation into the biosynthetic recombinant yeast steroids. The $\delta\ ^{13}C$ was very different from the natural human steroids one.

More recently, hydrocortisone and cortisone were also produced by other recombinant yeast (2). Corticosteroids could easily be studied by GC-C-IRMS as androstan-tri-one derivatives (3), and, as anabolic steroids, the differentiation between synthetic and natural human ones was effective. The choice of 11-hydroxy-etiocholanolone as an ERCs for anabolic steroids GC-

Dr. Makoto Ueki
Head of the IOC/WADA Laboratory
Mitsubishi Kagaku Bio-Clinical Laboratories, Inc.
Japan

Dr. Michael Verkouteren
Research Chemist
National Institute of Standards and Technology
United States

Prof. Christina Wang
Program Director
Harbor-UCLA Medical Center
United States

Mr. Rich Wanninger
Communications & Public Affairs Director
USADA
United States

Ms. Andrea Wickerham
Legal Relations and Policy Director
The National Center for Drug Free Sport
United States

Dr. Prof. Mountian Wu
Head of the IOC/WADA Laboratory
China Doping Control Center
China

Mr. Richard Young
USADA Counsel
Holme, Roberts & Owen, LLP
United States

Dr. Mario Zorzoli
Official Doctor
Union Cycling Internationale (UCI)
Switzerland

going to be very difficult. What if the athlete from Kenya lives in America on Mc Donald's for one month, two, three months, or one year? Is that going to change what the values are? It is really very difficult.

FOURCROY
We put too much emphasis on diet and not enough emphasis on natural mutations. We certainly know there are differences in androgen metabolic pathways in several countries, and I think looking at it from what causes cancer. I think we put much too much emphasis on the diet; although, we know some diet does change. I would hate to think we do not look at the metabolic pathways that are really sped up in some countries.

KAZLAUSKAS
I agree we have to do that. We do not understand that, we do not know what those effects are, but the other thing of interest is that understanding the Kenyan's eat a lot of corn.

BOWERS
So, again there is nothing wrong with taking a cutoff that identifies too many people because presumably you have got a confirmation method that then sorts that out. So having said that what is your confirmation method after you do this?

KAZLAUSKAS
In terms of confirmation, we would actually go back to repeating this sort of experiment. We would also have a look at diols and use some of their values, I guess, from the literature. Because we don't have the extensive studies on the diols, so, we actually look at a number of different things before we could do that.

BOWERS
Based on what Dr. Fourcroy said, no matter what we pick for criteria there is somebody in that population that is somewhat disadvantaged. If you have a TE ratio of 3.5 and that is natural you take a little bit of T, and you are over the 6 limit. If you are 0.1 you take a whole bunch of T and never get close, so I am not too concerned about the thing that you showed with the exogenous and endogenous. In fact, in a way you have actually reversed the population that are advantaged and disadvantaged.

KAZLAUSKAS
There are still a lot of false negatives. I think we are increasing the false negative results rather than the false positive. Again everyone is concerned about false positives, but I think the result of all of this is showing that we

USADA 0865

- There is a need for additional research funding in this area. Topics include the sensitivity and specificity of screening using A/E versus the Adiols.

Session 1:

Basics of GC-C-IRMS in Doping Control

Chair:

Don Catlin

USADA 0866

REFERENCES

1. Preliminary results on the carbon isotope ratios of ketonic steroids in urines collected from different countries, A. Cawley, J. Rogerson, K. Rahman, G. Trout, and R. Kazlauskas, Recent Advances in Doping Analysis (11), Proceedings of the Manfred Donike Workshop 21st Cologne Workshop on Dope Analysis, 16th to 21st March 2003, ed W. Schänzer *et al*, Sport & Buch Strauss, Köln 2003, p183-193.
2. Expression of Differences in Isotopic Composition, U. Flenker and W. Schanzer, Advances in Doping Analysis (11), Proceedings of the Manfred Donike Workshop 21st Cologne Workshop on Dope Analysis, 16th to 21st March 2003, ed W. Schänzer *et al*, Sport & Buch Strauss, Köln 2003, p179-182.

Question & Answer Discussion

KAZLAUSKAS
We have just simply taken **A**, naturally occurring within the individual at –24, and you administer a **T** at –30. Then you have 80% of the **A** in that individual is exogenous, you do just a proportionation calculation with what the delta values should be. So that gives you the calculated delta value for difference and elimination. You can see there is a very marked difference (variation) in the actual values for each of those proportions depending on what the starting **A** value is in that individual.

HOUGHTON
I would like to question the need for a cut off value of -27. To me, the value in this technique is that each individual acts as their own control. I can see, the value in the population base is that you are showing steroid in the biosynthetic pathway, all having similar values. From that point on, the value is in the difference between the ERC and the analyte of interest. So why do you need to say the cutoff at -27?

KAZLAUSKAS
I agree with you. I forgot to say was we had this as our criteria, but we are now saying the absolute cutoff is probably not the way to go and, in fact, a difference protocol is far more useful. We need to now work out what the difference is the best value. Until we have all the data in for 1200 or so elite athletes and the statistician has done his job, we will not know what the difference should be. How do you calculate that? Do you have to take in to account the country the individual came from, but that in itself is really

going to be very difficult. What if the athlete from Kenya lives in America on Mc Donald's for one month, two, three months, or one year? Is that going to change what the values are? It is really very difficult.

FOURCROY
We put too much emphasis on diet and not enough emphasis on natural mutations. We certainly know there are differences in androgen metabolic pathways in several countries, and I think looking at it from what causes cancer. I think we put much too much emphasis on the diet; although, we know some diet does change. I would hate to think we do not look at the metabolic pathways that are really sped up in some countries.

KAZLAUSKAS
I agree we have to do that. We do not understand that, we do not know what those effects are, but the other thing of interest is that understanding the Kenyan's eat a lot of corn.

BOWERS
So, again there is nothing wrong with taking a cutoff that identifies too many people because presumably you have got a confirmation method that then sorts that out. So having said that what is your confirmation method after you do this?

KAZLAUSKAS
In terms of confirmation, we would actually go back to repeating this sort of experiment. We would also have a look at diols and use some of their values, I guess, from the literature. Because we don't have the extensive studies on the diols, so, we actually look at a number of different things before we could do that.

BOWERS
Based on what Dr. Fourcroy said, no matter what we pick for criteria there is somebody in that population that is somewhat disadvantaged. If you have a TE ratio of 3.5 and that is natural you take a little bit of T, and you are over the 6 limit. If you are 0.1 you take a whole bunch of T and never get close, so I am not too concerned about the thing that you showed with the exogenous and endogenous. In fact, in a way you have actually reversed the population that are advantaged and disadvantaged.

KAZLAUSKAS
There are still a lot of false negatives. I think we are increasing the false negative results rather than the false positive. Again everyone is concerned about false positives, but I think the result of all of this is showing that we

- There is a need for additional research funding in this area. Topics include the sensitivity and specificity of screening using A/E versus the Adiols.

- The $\delta^{13}C$ values obtained for **A**, **E** and 11-keto were found by ANOVA analysis to be significantly different ($p<0.0001$) for Kenya, China and Australia/New Zealand.
- The distributions are close to normal with some differences in standard deviations for each country.
- There is a consistent difference of approximately 2‰ between the average of **A** and **E** and the ERC.
- The most negative $\delta^{13}C$ value found for **A** is –25.1‰ and for **E** is –25.8‰. Thus the absolute cutoff of –27.0‰ is supported.
- The use of ratio of $\delta^{13}C$ values to ERC is not supported as the ratio is dependent on the size of the values measured (1,2)
- Athletes with higher $\delta^{13}C$ values for the **A**, **E** and ERC are advantaged relative to those with lower values as more exogenous material is needed for them to fail all criteria (difference to ERC and absolute value). However they are more sensitive to the difference criteria alone.
- The absolute cutoff of –27.0‰ may be reasonable for Australian and New Zealand populations but may not be appropriate for populations with higher d13C values such as the Kenyan population. A possible general rule is to define doping as a difference of 6‰ or greater or a difference of 4‰ or greater providing the $\delta^{13}C$ values of **A** and **E** are more negative than -27.0‰.
- These values only apply if 11-ketoetiocholanolone is used as the ERC. Using pregnanediol or other ERC markers gives different values for the difference cutoff and population studies using those markers need to be performed.

Table summarizing the $\delta^{13}C$ data for the various countries.

Country	Number	Mean Etio ‰	Mean Andro ‰	Mean 11-keto ‰
Australia	59	-23.0 SD 0.97 Min -25.3	-22.5 SD 1.16 Min -24.6	-21.1 SD 1.29 Min -24.1
New Zealand	108	-23.3 SD 1.07 Min -25.8	-23.0 SD 1.16 Min -25.1	-20.9 SD 1.21 Min -23.8
China	157	-22.5 SD 1.29 Min -25.0	-21.6 SD 1.53 Min -25.0	-20.1 SD 1.59 Min -24.0
Kenya	127	-20.5 SD 1.53 Min -24.0	-20.3 SD 1.38 Min -23.0	-18.6 SD 1.81 Min -24.0

USADA 0867

Human Anabolic/Androgenic Steroid Synthesis and Metabolism

Richard Auchus, M.D.
University of Texas Southwestern Medical Center

Steroid Biosynthesis:

The Five Components of Steroidogenesis:

1. The conversion of cholesterol to pregnenolone. The mobilization of cholesterol into the steroidogenic pathways is a complex event that not only serves as a key locus of acute regulation but also conventionally defines a tissue as "steroidogenic"—the adrenal cortex, ovarian theca cells, and trophoblast cells of the placenta.
2. The transformation of pregnenolone to active hormones, intermediates and exported steroid derivatives. The repertoire of enzymes and cofactor proteins present in a given steroidogenic cell generates the characteristic steroid profile of that cell type, and the coordinate regulation of their expression promotes the completion of all steps of a given pathway. Thus, these enzymes determine qualitatively *what* steroids are made, but since these steps are not kinetically limiting, it is Step 1 that quantitatively regulates *how much* steroid is made at a given moment.
3. Peripheral metabolism of hormones and precursors. Although not "steroidogenic" as defined above, some organs like the liver and skin possess tremendous capacity to transform a variety of steroids. For example, ~80% of circulating **T** in normal menstrual cycle women derives from the conversion of adrenal DHEA.
4. Target tissue metabolism. Steroids can be activated in target tissues, such as the conversion of **T** by steroid 5α-reductase, type 2, to dihydro**T** in the prostate. In contrast, active androgens and estrogens are inactivated in the uterus and other peripheral tissues by 17 β hydroxysteroid dehydrogenase (17βHSD), type 2.
5. Catabolism and unproductive metabolism. A panopoly of steroids can be isolated from human plasma and tissues, many of which have negligible biological activity. Most inactive by-products derive from hepatic transformations, such as 6α/6β-hydroxylation of C_{19} steroids, which promote renal excretion of these steroids.

Types of Enzymes

Cytochromes P450. P450 enzymes activate molecular oxygen using their heme center and electrons from Nicotinamide-Adenine Dinucleotide Phosphate (NADPH) via "redox partners" (flavo- or

metallo-proteins). P450 reactions on steroids are limited to oxygen insertion (hydroxlyation) reactions and, in a few notable cases, oxidative carbon-carbon bond cleavage reactions. P450 reactions irreversibly oxidize and/or remove carbon atoms from the steroid nucleus, and thus an organized sequence of these reactions forms the traditional pathways of steroid synthesis.

Hydroxysteroid Dehydrogenases (HSDs) and Reductases. All HSDs and related enzymes use nicotinamide cofactors either to reduce or to oxidize the steroid by two electrons with two hydrogen atoms. Most examples involve the conversion of a secondary alcohol to a ketone or vice-versa, and in the case of the 3β-hydroxysteroid dehydrogenase/$\Delta^{5/4}$-isomerases (3βHSDs), the dehydrogenation is accompanied by the isomerization of the adjacent carbon-carbon double bond from the Δ^5 (between carbons 5 and 6) to the Δ^4 positions. A few members of this group, such as the steroid 5α- and 5β-reductases, reduce olefinic carbon-carbon double bonds to the saturated state rather than acting on oxygenated carbon centers.

The HSDs can be broadly dichotomized according to either structural or functional classifications. *Structurally*, HSDs are members of either the short-chain dehydrogenase reductase (SDR) or aldo-keto reductase (AKR) families. Human 17βHSDs types 1-3 are SDR enzymes, and they are efficient, selective, membrane-bound enzymes. The reductive 3α-HSDs and 17β-HSD5 are AKR enzymes, and these are relatively slow, promiscuous, and soluble proteins. *Functionally*, HSDs act either as true dehydrogenases, using Nicotinamide-Adenine Dinucleotide $(NAD)^+$ as cofactor to convert hydroxysteroids to ketosteroids, or as ketosteroid reductases, utilizing predominantly NADPH to reduce ketosteroids. The preferred direction of each enzyme is determined by cofactor binding, because cofactor concentrations exceed steroid concentrations by many orders of magnitude. The reactions catalyzed by key isoforms of these enzymes are shown in the Figures 1 & 2.

Peripheral Metabolism

Concepts

It is important to distinguish irreversible from reversible enzymatic transformations. Reversible processes dynamically influence steroid potency in the plasma and in cells. The end-products of steroid metabolism excreted in the urine are determined by the irreversible transformations that tend to drive steroids "downhill" to inactive

USADA 0868

Some preliminary results on the carbon isotope ratios of ketonic steroids in urines collected from different countries

Adam Cawley, Jill Rogerson, Kishwar Rahman, Graham Trout, and Ray Kazlauskas
Australian Sports Drug Testing Laboratory

This talk was given to provide some information on the variation of the Carbon Isotope Ratio for different countries. We measure the $\delta^{13}C$ values of **A** and **E** in urine as indicators of administration of exogenous source of an anabolic steroid which may also occur naturally. This procedure works best for the "prohormones" AED, DHEA and the androstenediols. It also is a good indicator of **T** use but mainly for recent application of the drug.

In early 2000 as part of the EPO2000 project blood and urine samples were collected from some 1200 elite athletes around the world. The ethics approval for this project included analysis of the urine samples for steroids and in particular CIR measurements. The use of these samples allows us to get a good insight into the variability of the CIR values for a number of countries where diet may be the major contributor to any variability (1).

We use an endogenous internal marker (ERC) which is a compound that occurs through a different metabolic pathway and which is not affected by administration of **T** or its immediate precursors. The internal marker (11-ketoetiocholanolone) thereby represents the natural value of the CIR for that individual and marked difference from that marker in a direction towards the CIR of synthetic steroids indicates application of a drug. This internal marker is useful as it often occurs in easily measurable amounts. Typical internal marker concentrations are from 30% to 50% of the **A** concentration using our sample preparation procedure. The data we have accumulated over the past years allows a set of criteria to be established in order to allow a sample to be reported as being outside the normal range:

- The difference between the average of $\delta^{13}C$ for **A** and **E** and the $\delta^{13}C$ for the 11-ketoetiocholanolone (11-keto) must be greater than 4‰.
- The $\delta^{13}C$ for **A** and **E** must be more negative than –27.0‰ (3).

The results obtained for the samples collected from Australia, New Zealand, China and Kenya give insight into the applicability of the above criteria and are presented here. A summary of the findings is set out in the table below.

The data can be summarized as follows:

- The $\delta^{13}C$ values obtained for **A**, **E** and 11-keto were found by ANOVA analysis to be significantly different ($p<0.0001$) for Kenya, China and Australia/New Zealand.
- The distributions are close to normal with some differences in standard deviations for each country.
- There is a consistent difference of approximately 2‰ between the average of **A** and **E** and the ERC.
- The most negative $\delta^{13}C$ value found for **A** is –25.1‰ and for **E** is –25.8‰. Thus the absolute cutoff of –27.0‰ is supported.
- The use of ratio of $\delta^{13}C$ values to ERC is not supported as the ratio is dependent on the size of the values measured (1,2)
- Athletes with higher $\delta^{13}C$ values for the **A**, **E** and ERC are advantaged relative to those with lower values as more exogenous material is needed for them to fail all criteria (difference to ERC and absolute value). However they are more sensitive to the difference criteria alone.
- The absolute cutoff of –27.0‰ may be reasonable for Australian and New Zealand populations but may not be appropriate for populations with higher d13C values such as the Kenyan population. A possible general rule is to define doping as a difference of 6‰ or greater or a difference of 4‰ or greater providing the $\delta^{13}C$ values of **A** and **E** are more negative than -27.0‰.
- These values only apply if 11-ketoetiocholanolone is used as the ERC. Using pregnanediol or other ERC markers gives different values for the difference cutoff and population studies using those markers need to be performed.

Table summarizing the $\delta^{13}C$ data for the various countries.

Country	Number	Mean Etio ‰	Mean Andro ‰	Mean 11-keto ‰
Australia	59	-23.0 SD 0.97 Min -25.3	-22.5 SD 1.16 Min -24.6	-21.1 SD 1.29 Min -24.1
New Zealand	108	-23.3 SD 1.07 Min -25.8	-23.0 SD 1.16 Min -25.1	-20.9 SD 1.21 Min -23.8
China	157	-22.5 SD 1.29 Min -25.0	-21.6 SD 1.53 Min -25.0	-20.1 SD 1.59 Min -24.0
Kenya	127	-20.5 SD 1.53 Min -24.0	-20.3 SD 1.38 Min -23.0	-18.6 SD 1.81 Min -24.0

Human Anabolic/Androgenic Steroid Synthesis and Metabolism

Richard Auchus, M.D.
University of Texas Southwestern Medical Center

Steroid Biosynthesis:

The Five Components of Steroidogenesis:

1. The conversion of cholesterol to pregnenolone. The mobilization of cholesterol into the steroidogenic pathways is a complex event that not only serves as a key locus of acute regulation but also conventionally defines a tissue as "steroidogenic"—the adrenal cortex, ovarian theca cells, and trophoblast cells of the placenta.
2. The transformation of pregnenolone to active hormones, intermediates and exported steroid derivatives. The repertoire of enzymes and cofactor proteins present in a given steroidogenic cell generates the characteristic steroid profile of that cell type, and the coordinate regulation of their expression promotes the completion of all steps of a given pathway. Thus, these enzymes determine qualitatively *what* steroids are made, but since these steps are not kinetically limiting, it is Step 1 that quantitatively regulates *how much* steroid is made at a given moment.
3. Peripheral metabolism of hormones and precursors. Although not "steroidogenic" as defined above, some organs like the liver and skin possess tremendous capacity to transform a variety of steroids. For example, ~80% of circulating **T** in normal menstrual cycle women derives from the conversion of adrenal DHEA.
4. Target tissue metabolism. Steroids can be activated in target tissues, such as the conversion of **T** by steroid 5α-reductase, type 2, to dihydro**T** in the prostate. In contrast, active androgens and estrogens are inactivated in the uterus and other peripheral tissues by 17 β hydroxysteroid dehydrogenase (17βHSD), type 2.
5. Catabolism and unproductive metabolism. A panopoly of steroids can be isolated from human plasma and tissues, many of which have negligible biological activity. Most inactive by-products derive from hepatic transformations, such as 6α/6β-hydroxylation of C_{19} steroids, which promote renal excretion of these steroids.

Types of Enzymes

Cytochromes P450. P450 enzymes activate molecular oxygen using their heme center and electrons from Nicotinamide-Adenine Dinucleotide Phosphate (NADPH) via "redox partners" (flavo- or

Session 4:

Harmonization of Inter-laboratory Results

Chair:

Richard Hilderbrand

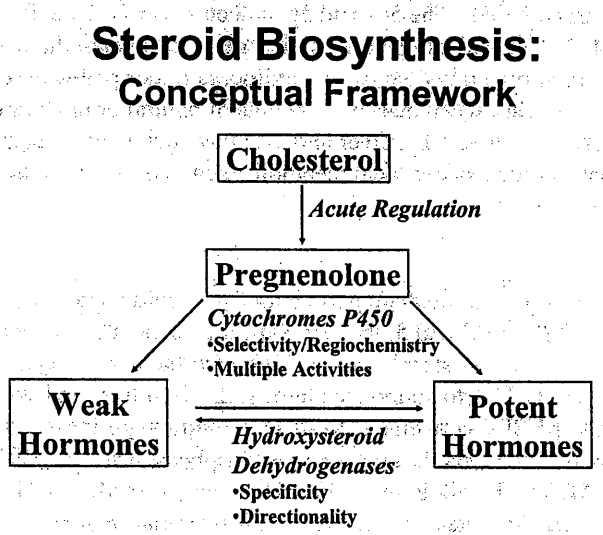
USADA 0869

compounds that are first oxidized (hydroxylated and/or cleaved by P450s) and then reduced (5α- or 5β-reduced 3, 17-hydroxysteroids). The 17βHSD and 3αHSD reactions are reversible, or at least have pairs of enzymes that preferentially run in the oxidative and reductive directions; the additional isomerization step renders the 3βHSD enzymes irreversible. The 5α- and 5β-reductases are irreversible, as are all P450-mediated oxygenations. Figures 1 & 2 detail the major pathways in normal physiology, but additional minor pathways can become significant when massive amounts of natural or unnatural steroids are consumed. The liver and skin have the greatest capacity to metabolize androgens, but local metabolism can also regulate hormone potency.

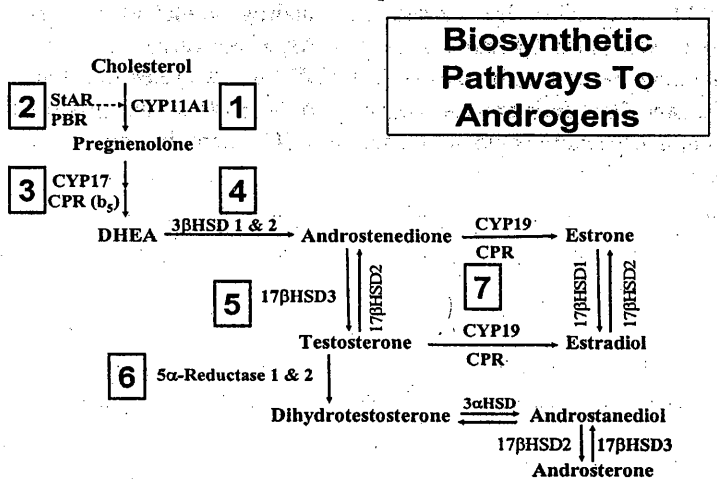
Enzymes

The key enzymes that mediate peripheral C_{19} steroid metabolism are 3βHSD1, 17βHSDs 2 and 5, the 3αHSDs, the 5α- and 5β-reductases, and CYP3A4. All Δ^5 steroids are converted to their Δ^4 congeners by 3βHSD1 (i.e. DHEA to androstenedione [AED]) in liver and skin. The oxidative 17βHSD2 converts **T** to AED in multiple peripheral tissues, and 17βHSD5 converts AED to T in the periphery, whereas 17βHSD3 converts AED to **T** only in the testis. These enzymes also metabolize AED and 5α-dihydrotestosterone (DHT) in a similar manner. Analogously, the oxidative and reductive 3αHSDs interconvert DHT and Adiol. The 5α- and 5β-reductases convert T to DHT and etiocholan-17β-ol-3-one, respectively. Finally, hepatic P450s, mainly CYP3A4, 6α- and 6β-hydroxylate many androgens such as **A**. For these reasons, many precursors (DHEA, AED, and variants on these structures) can be metabolized to T and DHT and excreted in the urine as 5α- and 5β-Adiols or as **A** and **E**. Also, 6α-hydroxy metabolites are common, especially when large amounts of C_{19} steroids are ingested orally.

Figure 1: (Top) The conceptual framework of how different enzymes and tissues participate in steroid biosynthesis and metabolism. Many aspects of the 5 components of steroidogenesis can all be incorporated into this scheme.



(Bottom) The principal enzymes involved in the biosynthesis of the active androgens T and DHT. Some subsequent products, the estrogens and A + Adiol, are included for completeness.



USADA 0870

The following points were emphasized:

- Well-separated peaks with good symmetry are important to analysis.
 - Contributions of system components to peak broadening and tailing
 - Potential for use of programmable temperature injection (PTI) to improve peak shape and increase amount of compound applied to the column
- There are differences between results based on instrumental approach to peak integration and calibration.
 - Importance of integration algorithm (e.g., summation)
 - Potential for application of peak fitting to improve results
 - Data reduction with a single algorithm independent of the instrument
- Characterization of peak envelope – width, height, resolution, shoulders, asymmetry – and development of criteria for acceptable peak characteristics are important.
- Differences between elemental analysis and GC-C-IRMS values for the same compound create concerns about purity.

Session 4:

Harmonization of Inter-laboratory Results

Chair:

Richard Hilderbrand

compounds that are first oxidized (hydroxylated and/or cleaved by P450s) and then reduced (5α- or 5β-reduced 3, 17-hydroxysteroids). The 17βHSD and 3αHSD reactions are reversible, or at least have pairs of enzymes that preferentially run in the oxidative and reductive directions; the additional isomerization step renders the 3βHSD enzymes irreversible. The 5α- and 5β-reductases are irreversible, as are all P450-mediated oxygenations. Figures 1 & 2 detail the major pathways in normal physiology, but additional minor pathways can become significant when massive amounts of natural or unnatural steroids are consumed. The liver and skin have the greatest capacity to metabolize androgens, but local metabolism can also regulate hormone potency.

Enzymes

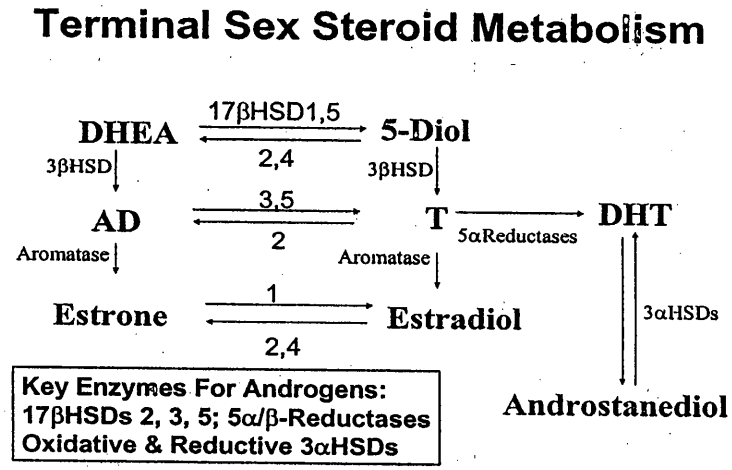
The key enzymes that mediate peripheral C_{19} steroid metabolism are 3βHSD1, 17βHSDs 2 and 5, the 3αHSDs, the 5α- and 5β-reductases, and CYP3A4. All Δ^5 steroids are converted to their Δ^4 congeners by 3βHSD1 (i.e. DHEA to androstenedione [AED]) in liver and skin. The oxidative 17βHSD2 converts **T** to AED in multiple peripheral tissues, and 17βHSD5 converts AED to T in the periphery, whereas 17βHSD3 converts AED to **T** only in the testis. These enzymes also metabolize AED and 5α-dihydrotestosterone (DHT) in a similar manner. Analogously, the oxidative and reductive 3αHSDs interconvert DHT and Adiol. The 5α- and 5β-reductases convert T to DHT and etiocholan-17β-ol-3-one, respectively. Finally, hepatic P450s, mainly CYP3A4, 6α- and 6β-hydroxylate many androgens such as A. For these reasons, many precursors (DHEA, AED, and variants on these structures) can be metabolized to T and DHT and excreted in the urine as 5α- and 5β-Adiols or as **A** and **E**. Also, 6α-hydroxy metabolites are common, especially when large amounts of C_{19} steroids are ingested orally.

Summary of Discussions:

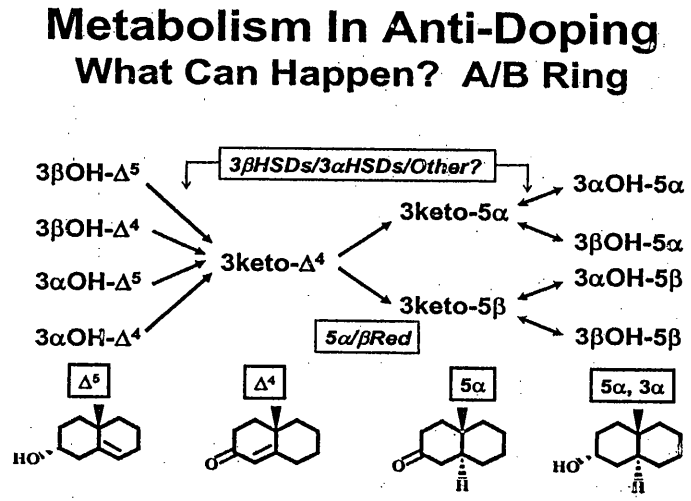
Uncertainty in GC-C-IRMS Measurement as Applied to Doping Control

USADA 0871

Figure 2: (Top) The HSD enzymes involved in the terminal stages of sex steroid (androgen and estrogen) biosynthesis and catabolism.



(Bottom) A generic scheme for the metabolic fates of C_{19} steroids with various A/B ring systems. The Δ^5 steroids can be metabolized to Δ^4 steroids by 3βHSD1.. The Δ^4 steroids are substrates for both the 5α- and 5β-reductases. Once the Δ^4 double bond has been saturated, these 3-ketosteroids can be further reduced, primarily by reductive 3αHSDs.



Question & Answer Discussion

COWAN
Thank you. It was a very interesting presentation. I would be very interested to hear your thoughts on the origin of epitestosterone (epi-T).

AUCHUS
I believe that it is an enzyme that we already know; we just don't know that it does it. It is probably one of the aldo-keto reductases; it may not be one of the aldo-keto reductase that we traditionally associate with steroid metabolism. There are now 240 aldo-keto reductase genes. This is one of the projects that Dr. Wilson and I have talked about doing. I would bet that it comes from 17 keto-steroid, there must be 3α-HSDs , and probably an aldo-keto reductase. Some of these aldo-keto reductases will be both 3α-HSDs and 20β –HSDs, so in other words, if you flip the steroid upside down it will work in the other position as well. So, it may be a 3β aldo-keto reductase that is doing 17α.

HALE
It is very good to hear interesting suppositions in a couple places there. Let's go back to the female. What happens if you overload in the female end organ system with the estrogen compound; are you saying that you can drive that back to **T** and that it would be a normal occurring **T**?

AUCHUS
No, no, no. The P450's are irreversible. With CYP19, which is an aromatase, it cleaves the C_{19} off. Once it is off it does not go back on. There are some compounds that have been said to have some androgenic activity that have an aromatic A ring, but the evidence is very weak. So you can covert androgens that have a C_{19} to estrogens, but you can't go back in the other direction. Some textbooks still talk about estrogen to androgen conversion, but it just doesn't happen.

Another question that is interesting in that regard. How do women make **T**? Because they do, and they do not have the type 3 enzyme. The type 3 enzyme is only found in the testes. Everybody thinks it is the type 5 enzyme, which I have already told you is a lousy enzyme. If you put the type 2 enzyme in the presence of AED, it will go back 2% to **T**. We actually think it is the type 2 enzyme that is supposed to inactivate it that is actually equilibrating a little percentage of it. If it is true, that

USADA 0872

performed gas chromatographic separation ideally resulting in base line separation, small peak widths and high peak symmetry. Furthermore the transfer of the analytes from the GC to the ion source via the open split and the combustion furnaces and the mass spectrometers themselves should be optimized where possible.

Choice of the method validation procedure

Validation of a method to International standards through a collaborative trial requires a method protocol to be followed very closely. The urinary studies are intended to provide such a method for validation by the primary partners in the ISOSTER project. However, extension of the validation to other laboratories is necessary according to the International standards Association of Analytical Communities (AOAC), International Union of Pure & Applied Chemistry (IUPAC).

As a consequence of this the attendees of the symposium are invited to encourage their laboratories to participate in the ISOSTER method validation study.

Question & Answer Discussion

UEKI
How does Dr. Preiss-Weigert express peak shape in writing? Do we have to calculate HETP in each run?

PREISS-WEIGERT
I cannot tell you. I have not designed the study, and I think they have not really defined the values for peak shape, which data they want to get back.

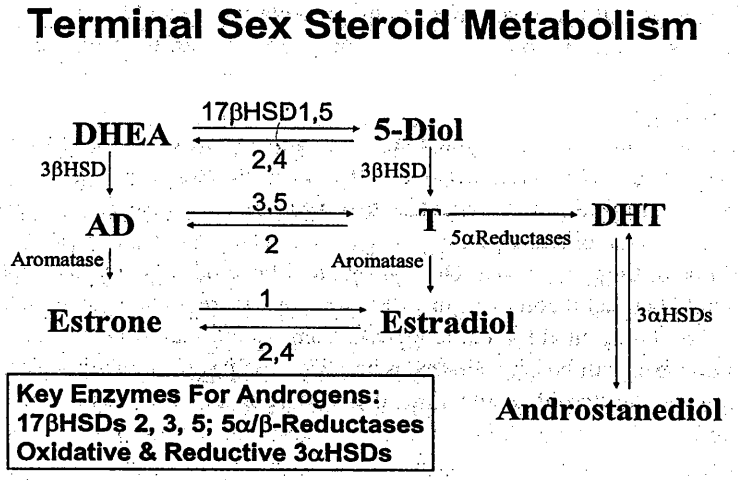
UEKI
The peak shape is very important, but I have no idea of how to express peak shape in writing.

PREISS-WEIGERT
The peak width of course and the symmetry of the peak, but they are the fundamentals.

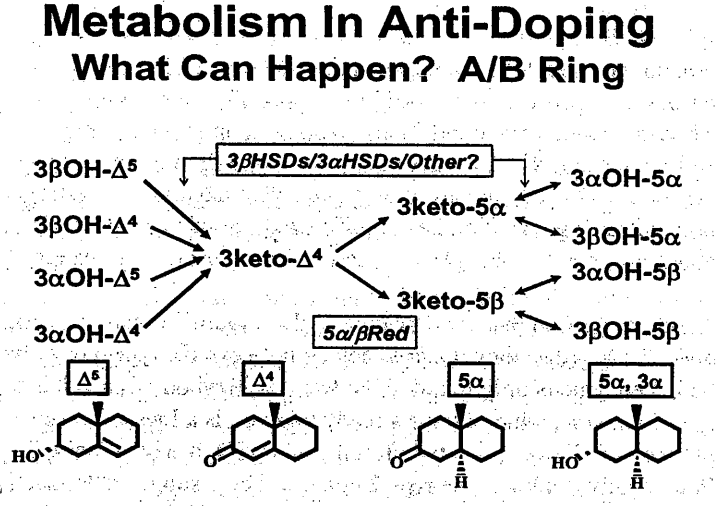
Summary of Discussions:

Uncertainty in GC-C-IRMS Measurement as Applied to Doping Control

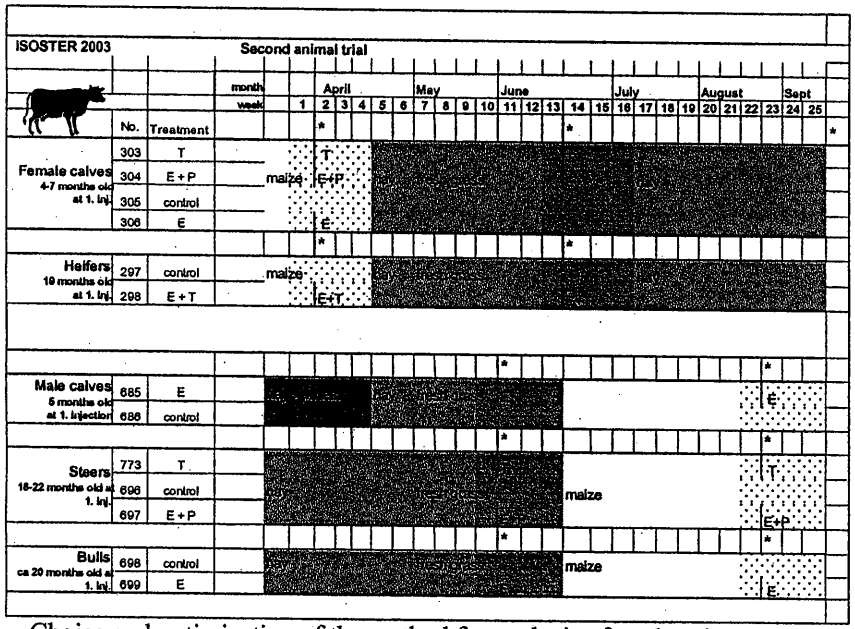
Figure 2: (Top) The HSD enzymes involved in the terminal stages of sex steroid (androgen and estrogen) biosynthesis and catabolism.



(Bottom) A generic scheme for the metabolic fates of C_{19} steroids with various A/B ring systems. The Δ^5 steroids can be metabolized to Δ^4 steroids by 3βHSD1. The Δ^4 steroids are substrates for both the 5α- and 5β-reductases. Once the Δ^4 double bond has been saturated, these 3-ketosteroids can be further reduced, primarily by reductive 3αHSDs.



Animal trial aiming at the comparison of the influences of different feeding regimen



Choice and optimization of the method for analysis of cattle urine

The project partners agreed to select the method developed by LABERCA, Nantes, Fr, as starting point for the method validation procedure. Optimization at several levels of the sample preparation is currently underway and involves the conditions of the deconjugation step, the SPE procedures, the HPLC purification system as well as the derivatization procedure.

The most interesting aspect will probably be the comparison of different types of derivatives. Matters for consideration will be possible fractionation effects during the derivatization reaction, their chromatographic properties, their signal intensities in the IRMS, the precision of the measured δ-values and finally their behaviour in a large volume injection system (PTV).

The quality of the measurements also depends upon the equipment and the skill in its application. Therefore the partners are supported by the manufacturers of the IRMS devices. Their input focuses on increasing the sensitivity of the measurement. Attention has to be paid to a thoroughly

USADA 0873

we can modulate that equilibrium to constant by modulating cellular cofactors, there can be conditions where more of it is generated by this very good enzyme that is found everywhere.

AGUILERA
How much evidence do you have of the transformation from Adiol to DHT?

AUCHUS
Of the oxidative 3α-HSDs, which can do that conversion, Adiol back to DHT, there are three in the human. Dr. David Russell has cloned one of those enzymes from prostate and genital skin. They are in the Retinol DeHydrogenase (RoDH) family. Because they are also retinal dehydrogenases; there are three of them. Dr. Stefan Anderson and I are starting to work on those as well. They are pretty efficient. They are certainly located in genital skin. Which is again, why we think this back door path to Adiol may be important during fetal development. How much capacity they have, we are not so sure of.

In the liver, the reductive 3α-HSDs dominate, which would convert DHT to Adiol. We can also make those enzymes go backwards by NADPH completion. We don't really have a good sense now of what the capacity of those enzymes to metabolize systemic loads of these steroids are. Certainly local amounts, there are plenty of capacity in the genital tissue. In terms of generating systemically large circulating pools; we don't really know the answer to that.

CATLIN
One of the things that sportsmen do is take big doses. They also take epi-T. Can you speculate what somebody would do if they took a huge dose of epi-T, either clinically or biochemically?

AUCHUS
If there were a 3α-HSD that can make it, then because, these enzymes really are reversible, you ought to go back to AED. Then that should proportionate between epi-T and T. I would think that the absolute amount of the T would go up. But how much relative to the Epi-T? Can you really use the ratio? That is why I am saying, we have to get a sense of abundance in addition to ratios here.

Fundamentals of compound identification and measurement using isotopes and mass spectrometry

Dwight Matthews, Ph.D.
University of Vermont

Detection of banned substances in vivo from blood, urine, or other physiological sampling falls into two areas: (i) detection of administered compounds that are not produced in the body (exogenous compounds administered exogenously, such as drugs) and (ii) detection of compounds that are produced in the body (endogenous compounds) that are administered exogenously. Detection of non-endogenous drugs is much simpler than detection of addition of endogenous compounds that are administered to produce a "drug" effect. Mass spectrometry has proven to be an extremely reliable, sensitive, and precise technique for measuring exogenous drugs in physiological samples. The primary techniques used are gas chromatography-mass spectrometry (GCMS) and, more recently, liquid chromatography-mass spectrometry (LCMS). These techniques form the basis for most measurement of drugs and drug metabolites in physiological samples.

Measurement of exogenously administered *endogenous* compounds is a much more difficult task. Because levels of most metabolites vary among individuals and metabolic state (e.g. fasting v. fed or time of day), simple measurement of an abnormally elevated concentration of a metabolite in the body or urine does not necessarily represent exogenous administration. Compounds with long half lives or which produce metabolites with long half lives are easier to define and detect on the basis of elevated concentrations. Compounds with rapid turnover in the body are difficult to detect.

An alternative approach is detecting exogenously administered compounds that exist normally as endogenous compounds using physicochemical differences between the exogenously administered and endogenously produced material. The physicochemical differences that are available are differences in natural abundance of isotopes in the two sets of compound that arise on the basis of the different synthetic pathways. Most exogenously administered compounds are made in laboratories; very few compounds are administered after isolation from an animal source. As such, the synthetic routes and the starting materials of synthesis are different from those produced endogenously.

USADA 0874

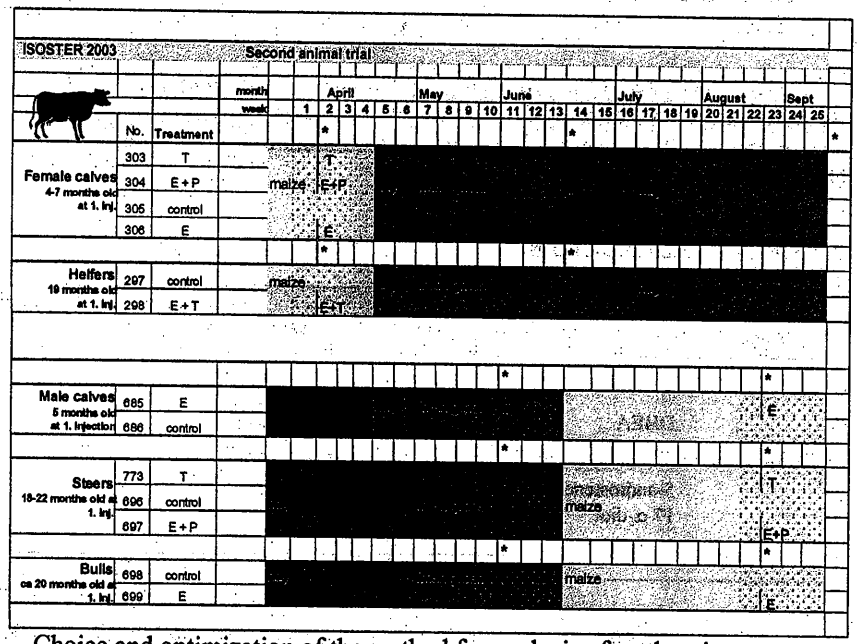
The presentation at this conference is focused on aspects of the ISOSTER project dealing with the analysis of urine samples. The presentation addresses the animal trials, the choice and the optimization of the analytical method and finally discusses the schedule of the method validation procedure.

Initially, the analytes for the measurements in urine had to be selected. The selection of the precursors and the target analytes depended on the concentration, which has to be in a sufficient amount to be measurable by GC-C-IRMS.

Selection of analytes for urine

Treatment	ERC	Target Analyte
EstrAdiol	DHEA	17 α-estrAdiol
	5-androstene-3 β, 17 α-diol	Estrone
T	DHEA	5 α-androstane-3 β,17 α-diol
	5-androstene-3 β, 17 α-diol	Etiocholanolone
		Epi-T

Animal trial aiming at the comparison of the influences of different feeding regimen



Choice and optimization of the method for analysis of cattle urine

The project partners agreed to select the method developed by LABERCA, Nantes, Fr, as starting point for the method validation procedure. Optimization at several levels of the sample preparation is currently underway and involves the conditions of the deconjugation step, the SPE procedures, the HPLC purification system as well as the derivatization procedure.

The most interesting aspect will probably be the comparison of different types of derivatives. Matters for consideration will be possible fractionation effects during the derivatization reaction, their chromatographic properties, their signal intensities in the IRMS, the precision of the measured δ-values and finally their behaviour in a large volume injection system (PTV).

The quality of the measurements also depends upon the equipment and the skill in its application. Therefore the partners are supported by the manufacturers of the IRMS devices. Their input focuses on increasing the sensitivity of the measurement. Attention has to be paid to a thoroughly

we can modulate that equilibrium to constant by modulating cellular cofactors, there can be conditions where more of it is generated by this very good enzyme that is found everywhere.

AGUILERA
How much evidence do you have of the transformation from Adiol to DHT?

AUCHUS
Of the oxidative 3α-HSDs, which can do that conversion, Adiol back to DHT, there are three in the human. Dr. David Russell has cloned one of those enzymes from prostate and genital skin. They are in the Retinol DeHydrogenase (RoDH) family. Because they are also retinal dehydrogenases; there are three of them. Dr. Stefan Anderson and I are starting to work on those as well. They are pretty efficient. They are certainly located in genital skin. Which is again, why we think this back door path to Adiol may be important during fetal development. How much capacity they have, we are not so sure of.

In the liver, the reductive 3α-HSDs dominate, which would convert DHT to Adiol. We can also make those enzymes go backwards by NADPH completion. We don't really have a good sense now of what the capacity of those enzymes to metabolize systemic loads of these steroids are. Certainly local amounts, there are plenty of capacity in the genital tissue. In terms of generating systemically large circulating pools; we don't really know the answer to that.

CATLIN
One of the things that sportsmen do is take big doses. They also take epi-T. Can you speculate what somebody would do if they took a huge dose of epi-T, either clinically or biochemically?

AUCHUS
If there were a 3α-HSD that can make it, then because, these enzymes really are reversible, you ought to go back to AED. Then that should proportionate between epi-T and T. I would think that the absolute amount of the T would go up. But how much relative to the Epi-T? Can you really use the ratio? That is why I am saying, we have to get a sense of abundance in addition to ratios here.

ISOSTER – Determination of the Origin of Hormones in Cattle

Angelika Preiss-Weigert[+1], Hildburg Fry[+1], Stefanie Banneke[+1], Bruno Le Bizec[+2], Wilhelm Schänzer[+3], Ed Houghton[+4] and Matthew Sharman[+5]

[+1] BfR, Berlin, D
[+2] LABERCA, ENV, Nantes, F
[+3] DSHS, Cologne, D
[+4] HFL, Fordham, UK
[+5] CSL, York, UK

The ISOSTER project is funded by the EC within the Growth programme as a part of the 5th framework programme. Four main partners (BfR, LABERCA, DSHS and CSL) are assisted by the Queens University in Belfast (QUB), UK, and by the TNO in Zeist, NL. Furthermore an added advantage is that the project involves three industrial partners: Thermo Finnigan, Bremen, D; GV Instruments, Manchester, UK; and DIONEX, Idstein, D.

The long term aim of the ISOSTER project is the creation of a tool for food control laboratories to allow them to control the administration of natural steroid hormones as growth promoting agents in food production.

The usual sampling locations of food control determine the choice of the matrix for analysis: as a consequence of this, methods applying GC-C-IRMS has to be developed for urine, feces, muscle, kidney, and fat samples. Additionally a major objective of this project is the validation of the GC-C-IRMS method for urine samples.

A further objective is the preparation of authentic samples derived from animals with a known history and kept under controlled conditions. In several animal trials, performed at the animal farm of the BfR in Berlin, samples from different categories of cattle (females and males, young animals and adults) have been collected. Thoroughly designed conceptual plans for the animal trials, taking into consideration the influences of feeding, should allow the variation of isotopic data of the steroids with and without administration of steroid hormones to be determined.

The aim of this project is to assess the effectiveness of the developed GC-C-IRMS methods for the differentiation between the endogenous and exogenous origin of steroids in cattle.

USADA 0875

Isotope geochemists and chemists studying organic reaction mechanisms have studied isotope effects of chemical reactions extensively. The isotope effects are generally proportional to the square root difference between the two isotopes being studied. For example, the isotope effect of deuterium from protium is in the range of $^{2}H/^{1}H = [2/1]^{½} - 1 = 41\%$ or more, while that of $^{13}C/^{12}C = [13/12]^{½} - 1 = 4\%$ or less. The isotope effects seen in nature approximate these differences in mass alone. Although significantly greater natural differences will be found for hydrogen, due to its larger isotope effects, use of isotope effects to detect exogenously given metabolites has centered on measuring isotope differences of carbon because methods of measurement of physiological samples have been developed more fully. Isotope ratio mass spectrometry (IRMS) was developed in the 1930's for measurement of light element isotopes and saw immediate application to measurement of isotopes of carbon, nitrogen, oxygen, and hydrogen.

The hydrogen isotopes have always been the most problematic to measure, while carbon isotopes of organic compounds have been the simplest because the material to be measured only has to be purified and oxidized to CO_2 for measurement by IRMS. The only drawback is that purification and isolation of a metabolite in small amounts from a physiological sample is not a simple task. In 1978 Matthews and Hayes, demonstrated a new technique that eventually came to be known as gas chromatography-combustion-IRMS (GC-C-IRMS). The technique put a GC as the front end of the mass spectrometer and inserted an oxidation oven between the GC and an IRMS to oxidize all material eluting from the GC to CO_2. This technique took advantage of the reduction of noise by measuring all ^{13}C as CO_2 and measuring all samples against a reference CO_2 gas of known isotopic content. Since the initial 1978 report, a number of developments (e.g. introduction of fused silica GC columns) made commercial instruments feasible, and many laboratories around the world now have GC-C-IRMS instruments as their primary approach to IRMS measurement.

The GC-C-IRMS measurement for carbon measures the $^{13}CO_2/^{12}CO_2$ ratio for eluting compounds from the GC against a standard reference CO_2 gas of known ^{13}C content. Thus, the common values reported from the GC-C-IRMS are ^{13}C values in delta (δ) units in per mil (‰):

$$\delta = [R/R_{std} - 1] \cdot 1000$$

where R is the $^{13}C/^{12}C$ ratio of the measured sample peak and R_{std} is the $^{13}C/^{12}C$ ratio of the accepted Vienna PeeDee Belemnite (VPDB) limestone standard (note: the actual δ that the instrument produces is referenced to a laboratory standard gas used for that particular instrument, then is referenced to the VPDB primary standard). Most practitioners express their results directly in δ units – even if these measurements do not have a direct connection to ^{13}C enrichment and are referenced to a non-physiological standard (limestone). The reason for staying with the δ unit is because the differences measured in ^{13}C abundance among samples are very small compared to natural abundance of ^{13}C. For example, the $^{13}C/^{12}C$ ratio of the VPDB standard is 1.12372, and a δ = 0.1‰ corresponds to a change in $^{13}C/^{12}C$ of = 0.00012 producing a new ratio equal to 1.12384. However, physiological expression of ^{13}C abundance can only be done in terms of enrichment – that is in terms of the amount of ^{13}C in excess of natural ^{13}C. These expressions require a mole fraction measurement of ^{13}C.

The first transformation to a mole fraction that needs to be done is to convert the δ unit to the ratio of $^{13}C/^{12}C$ in the sample:

$$R = (\delta /1000 + 1)R_{std}$$

The ratio of $^{13}C/^{12}C$ is then converted to the atom fraction of ^{13}C abundance (F): $F = R\ (1+R)$ which correspond to $F = {}^{13}C / ({}^{13}C+{}^{12}C)$. As per the $^{13}C/^{12}C$ ratio, expressing ^{13}C abundance as $^{13}C/(^{13}C+^{12}C)$ still produces a number that is large compared to the difference in enrichment measured *among* samples with IRMS. Thus, a "δ" equivalent is used by subtracting natural abundance of ^{13}C (F_0) from each measured sample's abundance (F). This expression is

$$E = F - F_0$$

where E is the abundance of ^{13}C above natural abundance. Because the differences expressed by E are small, the values are expressed in ppm of excess ^{13}C. Note also that a sample that contains presence of administered exogenous compound could produce an E value either positive or negative, depending upon the abundance of the ^{13}C in the exogenous material. In general, most synthetically derived compounds are isotopically lighter than endogenously synthesized material and E is generally negative.

USADA 0876

BRENNA
Correct, but the problem is solvable.

MATTHEWS
I want to make two points. One, better chromatography solves a host of problems, no question about that. Two, peaks that are buried under your peak are not going to be resolved by curve fitting or any other method whatsoever.

ISOSTER – Determination of the Origin of Hormones in Cattle

Angelika Preiss-Weigert[+1], Hildburg Fry[+1], Stefanie Banneke[+1], Bruno Le Bizec[+2], Wilhelm Schänzer[+3], Ed Houghton[+4] and Matthew Sharman[+5]

[+1] BfR, Berlin, D
[+2] LABERCA, ENV, Nantes, F
[+3] DSHS, Cologne, D
[+4] HFL, Fordham, UK
[+5] CSL, York, UK

The ISOSTER project is funded by the EC within the Growth programme as a part of the 5th framework programme. Four main partners (BfR, LABERCA, DSHS and CSL) are assisted by the Queens University in Belfast (QUB), UK, and by the TNO in Zeist, NL. Furthermore an added advantage is that the project involves three industrial partners: Thermo Finnigan, Bremen, D; GV Instruments, Manchester, UK; and DIONEX, Idstein, D.

The long term aim of the ISOSTER project is the creation of a tool for food control laboratories to allow them to control the administration of natural steroid hormones as growth promoting agents in food production.

The usual sampling locations of food control determine the choice of the matrix for analysis: as a consequence of this, methods applying GC-C-IRMS has to be developed for urine, feces, muscle, kidney, and fat samples. Additionally a major objective of this project is the validation of the GC-C-IRMS method for urine samples.

A further objective is the preparation of authentic samples derived from animals with a known history and kept under controlled conditions. In several animal trials, performed at the animal farm of the BfR in Berlin, samples from different categories of cattle (females and males, young animals and adults) have been collected. Thoroughly designed conceptual plans for the animal trials, taking into consideration the influences of feeding, should allow the variation of isotopic data of the steroids with and without administration of steroid hormones to be determined.

The aim of this project is to assess the effectiveness of the developed GC-C-IRMS methods for the differentiation between the endogenous and exogenous origin of steroids in cattle.

Isotope geochemists and chemists studying organic reaction mechanisms have studied isotope effects of chemical reactions extensively. The isotope effects are generally proportional to the square root difference between the two isotopes being studied. For example, the isotope effect of deuterium from protium is in the range of $^{2}H/^{1}H = [2/1]^{½} - 1 = 41\%$ or more, while that of $^{13}C/^{12}C = [13/12]^{½} - 1 = 4\%$ or less. The isotope effects seen in nature approximate these differences in mass alone. Although significantly greater natural differences will be found for hydrogen, due to its larger isotope effects, use of isotope effects to detect exogenously given metabolites has centered on measuring isotope differences of carbon because methods of measurement of physiological samples have been developed more fully. Isotope ratio mass spectrometry (IRMS) was developed in the 1930's for measurement of light element isotopes and saw immediate application to measurement of isotopes of carbon, nitrogen, oxygen, and hydrogen.

The hydrogen isotopes have always been the most problematic to measure, while carbon isotopes of organic compounds have been the simplest because the material to be measured only has to be purified and oxidized to CO_2 for measurement by IRMS. The only drawback is that purification and isolation of a metabolite in small amounts from a physiological sample is not a simple task. In 1978 Matthews and Hayes, demonstrated a new technique that eventually came to be known as gas chromatography-combustion-IRMS (GC-C-IRMS). The technique put a GC as the front end of the mass spectrometer and inserted an oxidation oven between the GC and an IRMS to oxidize all material eluting from the GC to CO_2. This technique took advantage of the reduction of noise by measuring all ^{13}C as CO_2 and measuring all samples against a reference CO_2 gas of known isotopic content. Since the initial 1978 report, a number of developments (e.g. introduction of fused silica GC columns) made commercial instruments feasible, and many laboratories around the world now have GC-C-IRMS instruments as their primary approach to IRMS measurement.

The GC-C-IRMS measurement for carbon measures the $^{13}CO_2/^{12}CO_2$ ratio for eluting compounds from the GC against a standard reference CO_2 gas of known ^{13}C content. Thus, the common values reported from the GC-C-IRMS are ^{13}C values in delta (δ) units in per mil (‰):

AUCHUS
To get rid of that little peak do you have to de-convolute or can you just do that with your single curve fitting?

BRENNA
Well, that is what you are doing. The word de-convolute is what is applied to what we are doing.

GEORGAKOPOULOS
If the software has the background subtraction algorithm, what is your opinion, is it useful to use and safe or not?

BRENNA
If you are thinking about a particular bit of software, I would not be able to address it specifically. All of the software has to subtract background; that is not optional. It cannot calculate an isotope ratio without subtracting background in some way.

BOWERS
Two points and see if you can synthesize these together. One, as you have gathered from this whole discussion the last couple of days, the sample matrix that we are dealing with contains a large number of substances, so the chances of having a really pure system are slim. Second, as you do this integration it is a serious issue to get de-convolated peaks. The problem I have is you are never going to standardize exactly between instruments, in particular, how this gets done. So what would your approach to this be? The summation issue is not going to go away, and you touched on the start and stop, which becomes even more important as you get overlapping things like that so where do we go from here?

BRENNA
An advantage to curve-fitting is that the start and stop points are not so important because they just specify a little more or less of the curve that is to be fit, and standardizing baselines and the possible overlaps is easier to handle. To answer the question in general, one would have to have of a general idea of what the range of expected chromatograms might be, including the typical contaminants.

BOWERS
Again, they are going to be sample specific, and so you are going to have to do something to deal with each sample.

USADA 0877

Natural abundance, F_0, will vary among individuals based upon dietary ^{13}C differences, etc. Thus, instead of using a fixed standard value for F_0, it is adjusted for each measurement set. Using an F_0 value established for each person measured allows definition of ^{13}C enrichment above (or depletion below) natural abundance to be established individually. The question is how do we obtain a natural abundance measurement of the amount of ^{13}C (i.e. F_0) present in a particular metabolite in a particular sample for a particular subject? Any measurement we want to make in a sample assumes that exogenous material may be present and the metabolite to be measured will be different from natural abundance ^{13}C. One approach is to define a reference value of natural abundance for the population being measured. Doing so requires that we assess various variables that might affect the ^{13}C natural abundance. These variables include diet, sex, and race. Diet would include people living in different parts of the world and consuming diets for different areas and different natural variations in ^{13}C abundance. While reference values could be established, there will be significant uncertainty associated with them. Adding further is the requirement that every lab accurately and precisely standardize their reference ^{13}C measurements to assure agreement in baseline ^{13}C abundances. The net effect becomes a diminished ability to detect changes in ^{13}C abundance due to increased windows of uncertainty in the reference ^{13}C values required to account for variability's.

An alternative approach is to take advantage of other metabolites that are generally present in the measurement of the test metabolite. Usually the synthetic pathways of the various metabolites that are measured have more than one route of synthesis, and a metabolite can be found that is typical, but unrelated to the test metabolite. The ^{13}C abundance of this typical, but unrelated metabolite can be used to define the reference ^{13}C abundance for each person measured. The added value of this approach is that many errors disappear. Errors in knowing exactly the reference $^{13}CO_2$ gas used to calibrate the instrument largely disappear because the test metabolite and the reference metabolite are measured with the same error offset, and the offsets disappear upon subtraction of one from the other. Likewise dietary and race differences are anticipated to affect equally the different metabolites with respect to ^{13}C abundance. Again these differences disappear when the ^{13}C abundance of the reference metabolite is subtracted from the test metabolite.

In summary, GC-C-IRMS measurement of isotopic abundance is a powerful tool to differentiate exogenously administered compounds that exist endogenously. The principal power of the technique is seen when measurements are expressed not as the archaic δ nomenclature, but as the fractional abundance of the measured isotope from natural abundance. This measurement is especially powerful when it can be combined with a determination of natural abundance of a metabolite in the sample that is related to the test metabolite, but is unaffected by the exogenous administration of the suspected drug. Although ^{13}C measurements are most commonly used, greater power could be found using ^{2}H isotope measurements because of the greater differences in ^{2}H isotope abundance compared to ^{13}C. Possibly the most powerful technique comes from combined ^{2}H and ^{13}C abundance measurements.

Question & Answer Discussion

AUCHUS
Is there anything unique about the Tert-Butyl Dimethyl Silyl Group (TBDMS) is it because it has the tert-butyl group? If you just put trimethyl silyl group (TMS) on, do you still see this?

MATTHEWS
We stopped at that point and said this is ridiculous. From a chromatographic point of view, the TMS derivative is not as nice and clean and stable, and is a more difficult, ugly thing to use, although it is a classic derivative. My anticipation is that you will see exactly the same thing. I think what happens is, one of the silicon carbon bonds never breaks. That forms the basis for the silicon carbide formation. If you look in the ceramics literature, you actually can find it. That silicon carbide is a nice ceramic, and it will do exactly what it does and describes. My presumption is that you find it with the TMS derivative as well.

BOWERS
If you read the literature, there is discussion about silicon actually poisoning the copper nickel catalyst that you use for combustions. Is that not a concern? Is this possibly an explanation for that poisoning? Or are the people who say that it is poison just not correct?

USADA 0878

more sophistication to look carefully and recognizes a good curve shape". At any rate, I do not know to what degree this has been incorporated in to other software.

VERKOUTEREN
In your curve fitting you mention there are four parameters to the curve. Are those determined empirically or exactly how were the parameters decided upon? Are they variable from instrument to instrument or from sample to sample?

BRENNA
They are variable from run to run. You can think the four parameters as exactly analogous to the two parameters you use for a calibration curve. With a linear regression, calculation of the slope and intercept is possible in closed mathematical form; so you can say the slope equals something and the intercept equals something else. Most curves cannot be modeled in closed form, as it turns out, so we use something called a Marquardt-Levenburg algorithm. It searches parameter space and finds a global minimum.

AUCHUS
You would think that if you have potential problems of small overlapping peaks, particularly if they are buried in your tails, that there might be some advantage of slicing the center cut of the peak. If you use your curve fitting you have to use the whole curve. If you use summation, I would think, that you could select what part of that curve you could sum. Where you did those experiments with the mixture, where you had low abundance that overlapped, a little bit, did you notice any difference in doing it those two ways?

BRENNA
First, using slices will not work because ^{13}C comes out a little bit earlier than ^{12}C, and so you have to integrate the whole peak to obtain an accurate isotope ratio. It is not like GCMS.

The rest of the paper is where we address the other cases, and evaluated a 1-10 and a 10-1 ratio of peak areas. If you have a 10-1 ratio, where you have a big peak first and a little one on the tail, curve fitting nicely recovers the big peak; I would not use the isotope ratio from little peak. The reason is that there is too much uncertainty in the shape of the baseline. The baseline in continuous flow is what drives everything.

AUCHUS
To get rid of that little peak do you have to de-convolute or can you just do that with your single curve fitting?

BRENNA
Well, that is what you are doing. The word de-convolute is what is applied to what we are doing.

GEORGAKOPOULOS
If the software has the background subtraction algorithm, what is your opinion, is it useful to use and safe or not?

BRENNA
If you are thinking about a particular bit of software, I would not be able to address it specifically. All of the software has to subtract background; that is not optional. It cannot calculate an isotope ratio without subtracting background in some way.

BOWERS
Two points and see if you can synthesize these together. One, as you have gathered from this whole discussion the last couple of days, the sample matrix that we are dealing with contains a large number of substances, so the chances of having a really pure system are slim. Second, as you do this integration it is a serious issue to get de-convolated peaks. The problem I have is you are never going to standardize exactly between instruments, in particular, how this gets done. So what would your approach to this be? The summation issue is not going to go away, and you touched on the start and stop, which becomes even more important as you get overlapping things like that so where do we go from here?

BRENNA
An advantage to curve-fitting is that the start and stop points are not so important because they just specify a little more or less of the curve that is to be fit, and standardizing baselines and the possible overlaps is easier to handle. To answer the question in general, one would have to have of a general idea of what the range of expected chromatograms might be, including the typical contaminants.

BOWERS
Again, they are going to be sample specific, and so you are going to have to do something to deal with each sample.

Natural abundance, F_0, will vary among individuals based upon dietary ^{13}C differences, etc. Thus, instead of using a fixed standard value for F_0, it is adjusted for each measurement set. Using an F_0 value established for each person measured allows definition of ^{13}C enrichment above (or depletion below) natural abundance to be established individually. The question is how do we obtain a natural abundance measurement of the amount of ^{13}C (i.e. F_0) present in a particular metabolite in a particular sample for a particular subject? Any measurement we want to make in a sample assumes that exogenous material may be present and the metabolite to be measured will be different from natural abundance ^{13}C. One approach is to define a reference value of natural abundance for the population being measured. Doing so requires that we assess various variables that might affect the ^{13}C natural abundance. These variables include diet, sex, and race. Diet would include people living in different parts of the world and consuming diets for different areas and different natural variations in ^{13}C abundance. While reference values could be established, there will be significant uncertainty associated with them. Adding further is the requirement that every lab accurately and precisely standardize their reference ^{13}C measurements to assure agreement in baseline ^{13}C abundances. The net effect becomes a diminished ability to detect changes in ^{13}C abundance due to increased windows of uncertainty in the reference ^{13}C values required to account for variability's.

An alternative approach is to take advantage of other metabolites that are generally present in the measurement of the test metabolite. Usually the synthetic pathways of the various metabolites that are measured have more than one route of synthesis, and a metabolite can be found that is typical, but unrelated to the test metabolite. The ^{13}C abundance of this typical, but unrelated metabolite can be used to define the reference ^{13}C abundance for each person measured. The added value of this approach is that many errors disappear. Errors in knowing exactly the reference $^{13}CO_2$ gas used to calibrate the instrument largely disappear because the test metabolite and the reference metabolite are measured with the same error offset, and the offsets disappear upon subtraction of one from the other. Likewise dietary and race differences are anticipated to affect equally the different metabolites with respect to ^{13}C abundance. Again these differences disappear when the ^{13}C abundance of the reference metabolite is subtracted from the test metabolite.

more dilute samples studied will have a negative excess of C13 over the standard. While this may be disconcerting to the reader, in any biological experiment what is really important is the difference in C13 concentrations in different compounds ..." *[emphasis in original]. A. O. C. Nier, In: Preparation and Measurement of Isotopic Tracers, 1946. Wilson, Nier, Riemann, Eds., p 28.*

2. B. F. Murphey, Phys. Rev. 1947, 72, 834–837.
3. K. J. Goodman and J. T. Brenna, Anal. Chem. 1994, 66(8) 1294-1301
4. K. J Goodman and J. T. Brenna, J. Chromatog A 1995, *689*, 63-68

Question & Answer Discussion

AGUILERA
Is Prof. Brenna looking at the curve fitting or using the summation?

BRENNA
Summation gave us errors but curve fitting did not, so curve fitting worked out nicely. If you have an overlap and you really, really, really, want to work out the isotope ratios, for instance you have a very valuable sample and you have just run it and there is no more, then overlaps can be handled with reasonable certainty.

MATTHEWS
When you say errors you mean inaccuracies?

BRENNA
Yes, an inaccuracy or bias.

AGUILERA
Do you know how many companies used the curve fitting approach?

BRENNA
No, these are all our algorithms. I do not know who has incorporated curve-fitting into their software. There has been talk over the years about incorporating it into software. It is not quite as straight forward as in the summation case because your curve shapes depend on your chromatography. I have some sympathy for the instrument manufacturers, in that they are trying to develop a machine that anybody can use for any curve shape. You get a number for any curve shape, but it takes a little bit

USADA 0879

MATTHEWS
There are worse poisons. Using a fluoro derivative, like a heptafluoro butyryl derivative, produces a wonderful poison called hydrogen fluoride. This eats away at the oxidation tube itself. But amazingly enough people actually use that derivative and use it fairly effectively. The silicon derivatives degrade, which I think is a better word than poison, so what they do is they lay down a combination of silicon dioxide, and in this case, silicon carbide, which is going to eventually coat and reduce the active surface area. Most practitioners in the field say that if I am going to have to change one of these tubes ever couple of months, then it is not so bad. I don't think anyone has seen these reductions of efficiencies occur overnight. They occur slowly, and it doesn't really reduce the lifetime of the tube significantly.

Session 2:

Application of GC-C-IRMS to Doping Control

Chair:

Larry Bowers

USADA 0880

dataset obtained from one vendor's IRMS and processed either with that vendor's algorithm or another vendor's algorithm. The results are shown in Figure 2 for about 20 fatty acid methyl esters. The overall precision of these analysis was SD ($\delta^{13}C$) <0.40‰. However

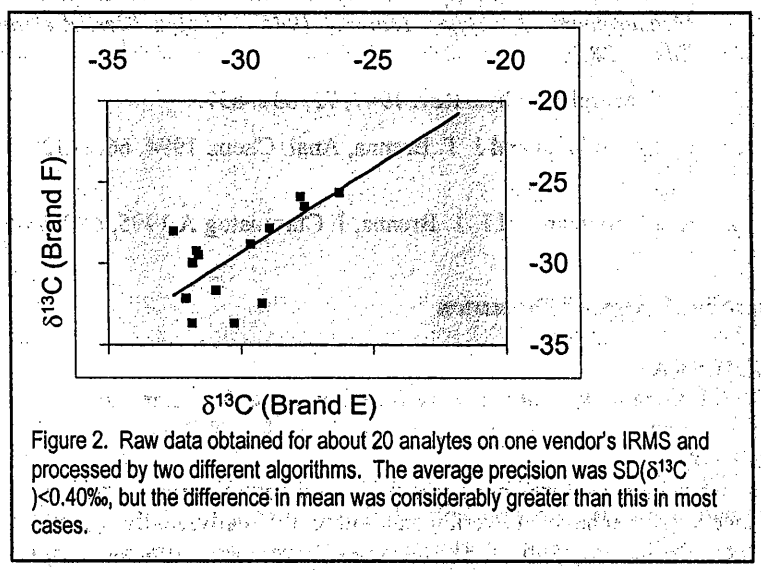


Figure 2. Raw data obtained for about 20 analytes on one vendor's IRMS and processed by two different algorithms. The average precision was SD($\delta^{13}C$)<0.40‰, but the difference in mean was considerably greater than this in most cases.

the root-mean-square difference in isotope ratios was about 2.1‰. It is important to emphasize that the raw data was identical; it was only the data reduction algorithms that differ.

We can conclude, overall, that the most reliable isotope ratio results are obtained when GC-C-IRMS peaks are strong and cleanly resolved from all other peaks. Though cross comparisons of algorithms are not common, insofar as they might be undertaken means isotope ratios may be unreliable for compounds with similar isotope ratios. Finally, these results demonstrate as dramatically as any, that *precision is no assurance of accuracy*, particularly in continuous flow IRMS. Good calibration standards are essential.

REFERENCES

1. "...it would seem highly advisable to always include in the published results the excess of tracer isotope ... above that found in some arbitrary standard. ... If ... ordinary chemical carbonate is used ... the normal average biological material will probably contain less C13 than does the laboratory standard and it and the

more dilute samples studied will have a negative excess of C13 over the standard. While this may be disconcerting to the reader, in any biological experiment what is really important is the difference in C13 concentrations in different compounds ..." *[emphasis in original]. A. O. C. Nier, In: Preparation and Measurement of Isotopic Tracers, 1946. Wilson, Nier, Riemann, Eds., p 28.*

2. B. F. Murphey, Phys. Rev. 1947, 72, 834–837.
3. K. J. Goodman and J. T. Brenna, Anal. Chem. 1994, 66(8) 1294-1301
4. K. J Goodman and J. T. Brenna, J. Chromatog A 1995, *689*, 63-68

Question & Answer Discussion

AGUILERA
Is Prof. Brenna looking at the curve fitting or using the summation?

BRENNA
Summation gave us errors but curve fitting did not, so curve fitting worked out nicely. If you have an overlap and you really, really, really, want to work out the isotope ratios, for instance you have a very valuable sample and you have just run it and there is no more, then overlaps can be handled with reasonable certainty.

MATTHEWS
When you say errors you mean inaccuracies?

BRENNA
Yes, an inaccuracy or bias.

AGUILERA
Do you know how many companies used the curve fitting approach?

BRENNA
No, these are all our algorithms. I do not know who has incorporated curve-fitting into their software. There has been talk over the years about incorporating it into software. It is not quite as straight forward as in the summation case because your curve shapes depend on your chromatography. I have some sympathy for the instrument manufacturers, in that they are trying to develop a machine that anybody can use for any curve shape. You get a number for any curve shape, but it takes a little bit

MATTHEWS
There are worse poisons. Using a fluoro derivative, like a heptafluoro butyryl derivative, produces a wonderful poison called hydrogen fluoride. This eats away at the oxidation tube itself. But amazingly enough people actually use that derivative and use it fairly effectively. The silicon derivatives degrade, which I think is a better word than poison, so what they do is they lay down a combination of silicon dioxide, and in this case, silicon carbide, which is going to eventually coat and reduce the active surface area. Most practitioners in the field say that if I am going to have to change one of these tubes ever couple of months, then it is not so bad. I don't think anyone has seen these reductions of efficiencies occur overnight. They occur slowly, and it doesn't really reduce the lifetime of the tube significantly.

Either summation or curve-fitting performs well for peaks represented by strong signals, flat and horizontal baselines, and excellent chromatographic resolution. Problems are encountered when peaks are close together or slightly overlapping, are of small signal, or appear on curved baselines (3). An example is shown in Fig 1. Two peaks of nearly identical $\delta^{13}C$ were progressively merged to yield varying degrees of overlap, and $\delta^{13}C$ calculated by summation or curve-fitting. Both algorithms produced excellent precision even in the presence of overlap as high as 40% valley. The summation algorithm means deviated from the known values, with the earlier eluting peaks apparently depleted, and the late eluting peak enriched. Curve-fitting is robust to overlap, maintaining good accuracy up to 40% valley. Superior performance has also been shown for curve-fitting in the case of low signal levels (4).

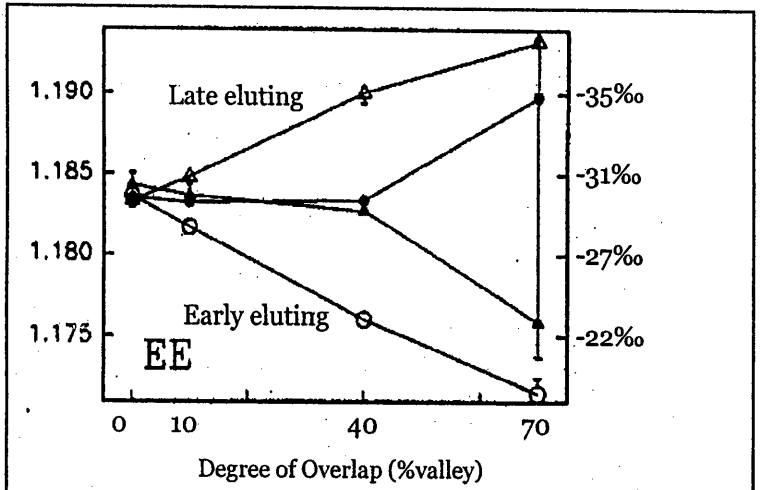


Figure 1. For data reduction by summation, the earlier eluting peak of a pair appears depleted while the later eluting peak appears enriched as a function of overlap. Curve-fitting does not suffer this problem until overlap is >40% valley. Left axis is R45 = I-45/I-44; right axis is $\delta^{13}C$. From Goodman and Brenna, Anal Chem 1994.

All algorithms do not result in the same isotope ratio when operating on the same data. We have recently observed a difference of about 0.7‰ when we mix summation and some forms of curve-fitting, though standardization against a single peak in a particular run removes this offset. A more dramatic example of this is found with a

USADA 0881

$^{13}C/^{12}C$-Measurement of 17-Ketosteroids to detect Abuse of Androgenic Steroids in Sport

Ulrich Flenker, Ute Gunter, and Wilhelm Schänzer
German Sports University

The two 17-keto-steroids **A** and **E** are the most abundant metabolites of androgenic steroids in humans. This applies to the hormones **T** and DHT as well as to pro-hormones such as DHEA, Δ5-AED, and Δ4-AED.

Synthetic steroids are produced predominantly by partial synthesis from phytosterols such as diosgenine, stigmasterine or sitosterine. Diosgenine is extracted from *Dioscorea* species, stigmasterine and sitosterine are extracted from soy. Due to their photosynthetic pathway these plants show a characteristic signature of the $^{13}C/^{12}C$-ratio. In case of administration of synthetic steroids this isotopic signature can be tracked forward to urinary excreted metabolites. Up to now only values have been reported that are significantly depleted in ^{13}C *vs.* endogenous steroids.

To detect the presence of exogenous steroids that contribute to androgenic metabolism the $\delta^{13}C$-values of the target compounds (TCs) **A** and **E** are compared to those of urinary steroids which are unrelated to this pathway. Such endogenous reference compounds (ERCs) are pregnanediol and 11-hydroxyAndrosterone.

To cope with analytical error on the one hand and with biological variation on the other, we established reference ranges (n = 60, students of physical education) for the isotopic composition of the TCs and the ERCs and for the isotopic differences between TCs and ERCs.

Table 1 shows the 99.9% reference limits and the corresponding 90% confidence intervals. With respect to differences between **A** and corresponding ERCs $\delta^{13}C_{PDB}$ -values of more than about 2.4 per mil are incompatible with sole presence of endogenous steroids assuming ^{13}C-depletion of the synthetic material. The values for **E** are somewhat larger, which we attribute to small isotopic fractionation during metabolism.

Anyway an apparent isotopic difference between **E** and **A** might also be due to chromatographic overlap. Although true baseline separation between **E** and **A** was achieved, we also calculated reference values for the differences between the ERCs and **E** and **A** integrated jointly. We hereby intended to construct an additional robust and integrative parameter.

The analytical method briefly is as follows:

1. Sample Preparation
 (a) 10 ml urine
 (b) SPE purification
 (c) Hydrolysis (β-glucuronidase, *E. coli*)
 (d) HPLC-purification, 3 fractions
 - Column: MERCK LiChrospher 100 RP-18, 200 × 4 mm, 5 µm
 - Flow: 1 ml/min
 - Solvent: H_2O/CH_3CN 70:30 ↦ 0:100 within 20 min
 - Automatic collection after standard measurement
 - **T** acetate as reference standard

2. GC Conditions
 (a) Hewlett Packard 5890-II
 (b) Column: Macherey & Nagel Optima-δ-3, 20 m × 0.25 mm, 0.25 µm film
 (c) Carrier gas: Helium (quality 5.0, additional gas purification), constant pressure at 30 psi
 (d) Injection: Splitless at 60°C, 1-3 µl (depending on concentration)
 (e) Temperature: 30°C/min ↦ 265°C, 3°C/min ↦ 295°C

3. Combustion Interface
 - FINNIGAN-MAT GC-Combustion Interface II, customized:
 (a) Metal X-piece replaced by Y-press-fit connector
 (b) Backflush valve moved outside the GC
 (c) Connection to reactor drilled up to 0.5 mm, capillary almost in touch with Cu/Ni/Pt wires
 (d) Reactor elevated about 1.5 cm, **mandatory** for measurement of free steroids

USADA 0882

Effects of Chromatographic Overlap on Uncertainty

Tom Brenna
Cornell University

Isotope ratio mass spectrometry (IRMS) is the method by which the most abundant isotopes of the organic elements are measured with the highest precision. The relevant elements are C, H, O, N, and S. The IRMS instrument is optimized to measure, for instance, CO_2, for $^{13}C/^{12}C$ and $^{18}O/^{16}O$ with highest sensitivity to produce the best counting statistics for highest precision. This optimization comes at the expense of flexibility; all analytes must be converted to CO_2 prior to admission to the IRMS. Measurement to measurement precision expressed as coefficient of variation is around 0.03%. To limit the redundancy in reporting of results, and to emphasize the practice of measuring isotope ratios *relative* to a standard, the PDB notation was introduced as first outlined by Nier (1), $\delta^{13}C$, as the difference in isotope ratio between an international standard carbonate and the sample, in parts per thousand.

The original method for introducing sample into the instrument is due to Murphey (2), and is called the "dual inlet", referring to the use of two volumes for CO_2 gas, one for sample, the other for standard. This method yields raw data that is approximately constant and isotope ratios are easy to calculate from the raw data. More recent methods are called "continuous flow", because helium gas sweeps the analyte into the ion source. GC-C-IRMS is the prototypical technique in this category. Data reduction, the conversion of raw data into isotope ratios, is not straightforward in this technique because the signal is constantly changing. The analyte peak must be defined, integrated, and background subtracted to obtain the raw isotope ratio, and this must all be done with reproducibility quoted above. Several algorithms exist for this, and the "summation" algorithm is probably the most widely used. Here, a peak start and stop are defined by some criteria related to signal slope, background points are determined, and subtracted. An alternative is "curve-fitting", whereby the signal is modeled by a mathematical function that has the shape of a skewed bell-shaped curve. At least eighty different functions have been used to model chromatographic peaks. An example is the exponentially-modified Gaussian, which is a mathematical convolution of an exponential decay curve and a Gaussian.

Either summation or curve-fitting performs well for peaks represented by strong signals, flat and horizontal baselines, and excellent chromatographic resolution. Problems are encountered when peaks are close together or slightly overlapping, are of small signal, or appear on curved baselines (3). An example is shown in Fig 1. Two peaks of nearly identical $\delta^{13}C$ were progressively merged to yield varying degrees of overlap, and $\delta^{13}C$ calculated by summation or curve-fitting. Both algorithms produced excellent precision even in the presence of overlap as high as 40% valley. The summation algorithm means deviated from the known values, with the earlier eluting peaks apparently depleted, and the late eluting peak enriched. Curve-fitting is robust to overlap, maintaining good accuracy up to 40% valley. Superior performance has also been shown for curve-fitting in the case of low signal levels (4).

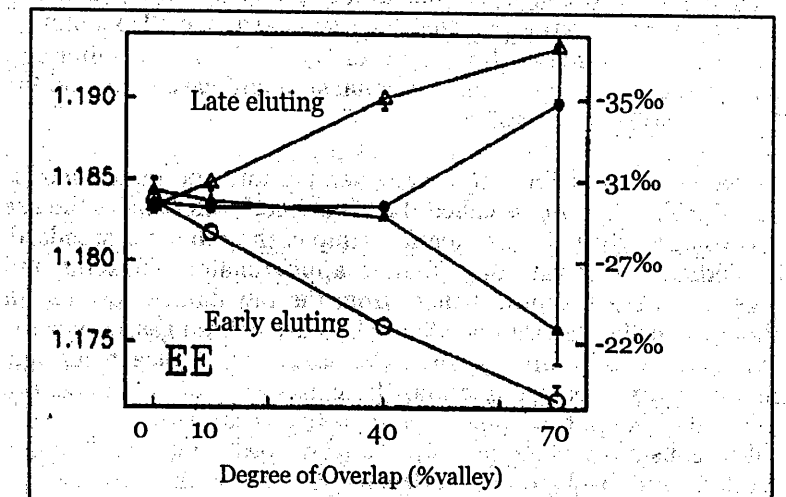


Figure 1. For data reduction by summation, the earlier eluting peak of a pair appears depleted while the later eluting peak appears enriched as a function of overlap. Curve-fitting does not suffer this problem until overlap is >40% valley. Left axis is R45 = I-45/I-44; right axis is $\delta^{13}C$. From Goodman and Brenna, Anal Chem 1994.

All algorithms do not result in the same isotope ratio when operating on the same data. We have recently observed a difference of about 0.7‰ when we mix summation and some forms of curve-fitting, though standardization against a single peak in a particular run removes this offset. A more dramatic example of this is found with a

$^{13}C/^{12}C$-Measurement of 17-Ketosteroids to detect Abuse of Androgenic Steroids in Sport

Ulrich Flenker, Ute Gunter, and Wilhelm Schänzer
German Sports University

The two 17-keto-steroids A and E are the most abundant metabolites of androgenic steroids in humans. This applies to the hormones T and DHT as well as to pro-hormones such as DHEA, Δ5-AED, and Δ4-AED.

Synthetic steroids are produced predominantly by partial synthesis from phytosterols such as diosgenine, stigmasterine or sitosterine. Diosgenine is extracted from *Dioscorea* species, stigmasterine and sitosterine are extracted from soy. Due to their photosynthetic pathway these plants show a characteristic signature of the $^{13}C/^{12}C$-ratio. In case of administration of synthetic steroids this isotopic signature can be tracked forward to urinary excreted metabolites. Up to now only values have been reported that are significantly depleted in ^{13}C *vs.* endogenous steroids.

To detect the presence of exogenous steroids that contribute to androgenic metabolism the $\delta^{13}C$-values of the target compounds (TCs) A and E are compared to those of urinary steroids which are unrelated to this pathway. Such endogenous reference compounds (ERCs) are pregnanediol and 11-hydroxyAndrosterone.

To cope with analytical error on the one hand and with biological variation on the other, we established reference ranges (n = 60, students of physical education) for the isotopic composition of the TCs and the ERCs and for the isotopic differences between TCs and ERCs.

Table 1 shows the 99.9% reference limits and the corresponding 90% confidence intervals. With respect to differences between A and corresponding ERCs $\delta^{13}C_{PDB}$ -values of more than about 2.4 per mil are incompatible with sole presence of endogenous steroids assuming ^{13}C-depletion of the synthetic material. The values for E are somewhat larger, which we attribute to small isotopic fractionation during metabolism.

programs you gravitate toward a mean. This is a fairly well known phenomenon. What I am trying to understand is the role you or NIST plays and to introduce this data management handling process into it. I am trying to figure out whether or not you just had a group of labs that worked hard, as you described, and did better over time. Therefore, the results got better, or how much of the contribution was because you were able to devise a central data management handling system.

VERKOUTEREN

The first inter-comparison was done with a very different set of labs. There were only maybe two labs that were common to both so the improvement in the results cannot be attributed to understanding the sample better. That was not it at all. Then, because we asked for measurement results rather than $\delta^{13}C$ results, they could not have possibly gotten better by knowing anything about those materials. Each laboratory has their own working reference gas, so they have their own delta measurements. In fact the composition of the working reference gas is totally immaterial in the inter-comparison because we did not reference to the working reference gas, but we referenced to another sample in the sequence. We only use the working reference gas to correct for drift, but the sample and standard all go through the same inlet. If there are any kinds of differences, in fractionation between inlets, they are corrected for in that way. Plus the samples were all of the same size, so they are all size matched. That was another reason why this inter- comparison may have had better results...it was more rigorously designed.

What you are trying to do is extremely challenging. You will find the first time around will be very discouraging. You will find you are way, way off having any kind of harmony, which is totally natural. It is going to take some time and resources, and it is going to require a lot of commitment to be able to reach harmony. I do not think it has really been done to this scale anywhere worldwide, and I am willing to help as much I can. It is going to take everyone's cooperation, everyone's knowledge and trust, to be able to achieve that. It is doable. It is just going to take a lot of effort.

USADA 0883

(e) Reduction furnace unmounted
(f) Nafion water trap replaced by cryotrap (Dewar, acetone/dry ice)

All compounds are measured in free form. Provided the GC-C system is in good condition this does not show any significant disadvantages compared to acetylation or silylation. On the other hand this circumvents possible problems with incomplete reaction and with blurring of isotopic signatures.

Table 1: Reference limits (99.9%) and corresponding confidence intervals (CI, 90%) for relevant $\delta^{13}C$-values. A: Androsterone; E: etiocholanolone; EA: E and A, common integration; PD: pregnanediol; 11OHA: 11-hydroxyAndrosterone.

	Reference Limits		
$\Delta\delta^{13}C$	Lower	Upper	CI
PD - E	-1.01	2.88	0.35
PD - A	-2.22	1.89	0.37
PD - EA	-1.70	2.27	0.36
11OHA - E	-1.49	3.73	0.49
11OHA - A	-1.84	1.83	0.34
11OHA - EA	-1.46	2.36	0.36

Applying GC-C-IRMS to samples that exhibited unusual steroid profiles, we were able to show presence of exogenous steroids as well as presumed absence.

Table 2 shows values of a sample reported negative: Especially the Testosterone/Epitestosterone (T/E) ratio and the T concentration are unusually high. Although these parameters might indicate T administration, $^{13}C/^{12}C$-ratios suggest physiological reasons for this observation.

Table 2: Sample 30638/2002: T/E = 8.1, A/T = 27, c_T = 157 ng/ml. Sample reported negative

Compound (n = 1)	$\delta^{13}C_{PDB}$
A	-23.0
E	-24.1
11OHA	-24.3

Table 3 conversely describes a sample that is mostly unsuspicious with respect to T. Yet the A/E ratio is unusual low, might indicate application of steroids that preferably are metabolized to E. Indeed isotopic data clearly show depleted $\delta^{13}C$-values. E is affected more than A. The whole pattern can be explained by administration of Δ5-pro-hormones.

Table 3: Sample 1145/2003: T/E = 4.9, A/E = 0.22, c_T = 5.3 ng/ml. Sample reported positive

Compound (n = 3)	$\delta^{13}C_{PDB}$
A	-26.4
E	-29.7
11OHA	-20.1
PD	-21.4

Question & Answer Discussion

AGUILERA
Do you know what amount of urine they used? You never used less than 10ml?

USADA 0884

Question & Answer Discussion

CATLIN
The experiment was very interesting that Dr. Verkouteren described, where a group of labs got together and did measurements, and then sent data through the algorithm that he described. He was pleased to find no outliers. Was that in the second trial? He must have specified outliers in the first go around or did the algorithm remove them all?

VERKOUTEREN
I only got $\delta^{13}C$ and $\delta^{18}O$ results for the CO_2. I had no information about what algorithms were used, I had no basis for throwing out any outliers and all the data you saw was all the data I collected for the first trial. For the second trial, I did not collect $\delta^{13}C$. I only collected the $\delta^{45}CO_2$ and $\delta^{46}CO_2$ and then a lot of information about the discretionary factors. Once I went through the algorithm and all the data handling in a very consistent way, I only had one outlier from a 100 results. That particular outlier had a procedural explanation. That was for VSMOW, the $\delta^{18}O$ of VSMOW. That particular lab used much too little water in the equilibration with a CO_2, so they were not following the best practice. That was identified and information transmitted back to them. They learned that fact from this inter-comparison.

Something else I always do is to make the reported inter-comparison results anonymous, so they are not linked with any particular lab. I respect the privacy of the lab, I do not want them to be embarrassed in anyway, and so I need enough labs so that the pool of data is large. Each lab gets to know who they are, of course, privately so they know what to then work on.

There is a NIST Report that came out on this, which the labs have had for about a year now. I get comments back when, after the labs find that, "oh my gosh, I did something wrong". They would like me to post-correct their data, but I go through that in the beginning. If I find something in the database that they report that just does not seem right, I will immediately come back and say, "is this right, is it a typo, or what is going on?" They have to verify to me that these particular values that they have reported are correct. At that point, they are hardened into stone, and then I go forth with the data evaluation.

CATLIN
This group does studies like that but not necessarily on carbon isotope ratio. We get a group of labs who participate in some sort of round robin, and we have seen over many, many years that if you participate in these PT

programs you gravitate toward a mean. This is a fairly well known phenomenon. What I am trying to understand is the role you or NIST plays and to introduce this data management handling process into it. I am trying to figure out whether or not you just had a group of labs that worked hard, as you described, and did better over time. Therefore, the results got better, or how much of the contribution was because you were able to devise a central data management handling system.

VERKOUTEREN

The first inter-comparison was done with a very different set of labs. There were only maybe two labs that were common to both so the improvement in the results cannot be attributed to understanding the sample better. That was not it at all. Then, because we asked for measurement results rather than $\delta^{13}C$ results, they could not have possibly gotten better by knowing anything about those materials. Each laboratory has their own working reference gas, so they have their own delta measurements. In fact the composition of the working reference gas is totally immaterial in the inter-comparison because we did not reference to the working reference gas, but we referenced to another sample in the sequence. We only use the working reference gas to correct for drift, but the sample and standard all go through the same inlet. If there are any kinds of differences, in fractionation between inlets, they are corrected for in that way. Plus the samples were all of the same size, so they are all size matched. That was another reason why this inter- comparison may have had better results…it was more rigorously designed.

What you are trying to do is extremely challenging. You will find the first time around will be very discouraging. You will find you are way, way off having any kind of harmony, which is totally natural. It is going to take some time and resources, and it is going to require a lot of commitment to be able to reach harmony. I do not think it has really been done to this scale anywhere worldwide, and I am willing to help as much I can. It is going to take everyone's cooperation, everyone's knowledge and trust, to be able to achieve that. It is doable. It is just going to take a lot of effort.

(e) Reduction furnace unmounted
(f) Nafion water trap replaced by cryotrap (Dewar, acetone/dry ice)

All compounds are measured in free form. Provided the GC-C system is in good condition this does not show any significant disadvantages compared to acetylation or silylation. On the other hand this circumvents possible problems with incomplete reaction and with blurring of isotopic signatures.

Table 1: Reference limits (99.9%) and corresponding confidence intervals (CI, 90%) for relevant $\delta^{13}C$-values. A: Androsterone; E: etiocholanolone; EA: E and A, common integration; PD: pregnanediol; 11OHA: 11-hydroxyAndrosterone.

	Reference Limits		
$\Delta\delta^{13}C$	Lower	Upper	CI
PD - E	-1.01	2.88	0.35
PD - A	-2.22	1.89	0.37
PD - EA	-1.70	2.27	0.36
11OHA - E	-1.49	3.73	0.49
11OHA - A	-1.84	1.83	0.34
11OHA - EA	-1.46	2.36	0.36

Applying GC-C-IRMS to samples that exhibited unusual steroid profiles, we were able to show presence of exogenous steroids as well as presumed absence.

RECOMMENDATIONS

The following recommendations are offered to help achieve the goal of international harmonization in GC-C-IRMS performance and results:

1. Identify specific analytical targets and acceptable limits of measurement uncertainty that are based on tolerable risks of false negative and false positive results.
2. Develop a suitable urine RMs, covering the range of observed steroid isotopic values, and assign documented isotopic values and uncertainties that are traceable to VPDB.
3. Develop performance-based standards to assess data quality through intercomparisons and proficiency tests.
4. In comparing two steroid measurements for a significant difference ($\Delta\delta$), these measurements should be performed under identical conditions and referenced to a RM to establish traceability to VPDB. Standard uncertainties of these two measurements can be used in a two-sided normal test (e.g., Natrella, 1963, Section 3-3.1.3) to determine whether the mean results are statistically distinct at a specified confidence level.

REFERENCES

1. Craig, H. (1957) *Geochim. Cosmochim. Acta* **12**, 133-149.
2. Eurochem/CITAC (2000) Guide to Quantifying Uncertainty in Analytical Measurement
3. Hut, G. (1987) Report of IAEA Consultants' group meeting on stable isotope reference samples
4. ISO-BIPM (1993) Guide to the Expression of Uncertainty in Measurement
5. ISO/IEC 17025 (1999) Requirements for the Competence of Calibration and Testing Laboratories
6. ISO-VIM (1993) International Vocabulary of Basic and General Terms in Metrology
7. Natrella, M. G. (1963) Experimental Statistics, NBS Handbook 91 (Reprinted 1966)
8. NIST SP260-149 (2003) Value Assignments and Uncertainty Estimation of Selected Light Stable Isotope Materials

USADA 0885

THEVIS
I think it was 2ml up to 20 ml. The average was 10 ml. So if we look up our standard operations procedures, it actually depends on the density and the amount of urine that was actually available. The average is approximately 10ml.

AGUILERA
Ten milliliters of urine is just about 20,000 ng of andro/etio. If you use an HPLC purification, do you know if there were some fractionation? When you do purification by HPLC, it is easy to have isotopic fractionation.

THEVIS
That is the reason why the fractions are pretty broad. As you saw the fractions were not tiny, and you could observe this phenomenon if they were smaller. That is why the fraction is about 2 minutes although the peak width is only 20 seconds, just for example. We need these high amounts because we focus our instrument to maximum linearity, so we sacrifice the sensitivity. So we try to compensate these things.

KAZLAUSKAS
You mentioned that you do a screening first without HPLC. Do you do the HPLC only for confirmation?

THEVIS
That is right.

HOUGHTON
How do you account for variation in the difference between ERC and A/E? You got that slight difference of 1, how do you account for that?

THEVIS
It is a well known phenomenon that you will have this difference. It occurs in basically every sample. So we didn't find that it was only with our reference population but basically every sample that we analyzed. That the E delta values are different by approximately 1 compared to A.

HOUGHTON
The enzyme process seems to be discriminating between the two stable isotopes.

BOWERS
You said you swapped out the Nafion water trap to the Cryotrap. Was there some reason for that specifically?

THEVIS
The Cryotrap was much more reliable than the Nafion trap. We had serious problems with the membrane. He said he had to exchange it every now and then. So he went over to use the Cryotrap which is easily regenerated by heating with a heat gun for a couple of minutes, and it was up and running again.

USADA 0886

While the example above deals with dual-inlet IRMS rather than GC-C-IRMS, these techniques share most components in the TMP (Fig.1). For doping control, the differences consist in the steps of sampling, stabilization, and chemical separation/purification. Admittedly, these steps most likely contain the greatest sources of measurement uncertainty, but the construct of the TMP allows the uncertainty budget to be expanded to account for these sources. Table 1 shows such a measurement traceability chain and estimated uncertainty budget.

Measurement Chain – Sample	Link in chain	Documentation	$\delta^{13}C$ value assignment	Uncertainty of Measurement Link (u_c)	Total Uncertainty to VDPB (u_c)
Matrix RM	5	Lab Report	Measurand	0.06 ‰ (est.)	0.12 ‰ (est.)
RM 8562 CO_2	4	TBD	TBD	0.10 ‰ (est.)	0.11 ‰ (est.)
NBS 19 limestone	3	NIST SP260-149 (2003)	-3.76 ‰	0.04 ‰	0.04 ‰
VPDB	2	Hut (1987)	+1.95 ‰	exact	0
SI	1	Craig (1957)	R = 0.0112372	0.0000300	(2.7 ‰)

Table 1: Traceability Chain and Estimated Uncertainty Budget for GC-C-IRMS Measurements

This chain of comparisons with traceability to VPDB presumes the development and use of a steroid isotope RM in a urine matrix with an uncertainty assigned through exhaustive type A and type B evaluations. Here, this combined uncertainty is estimated as 0.10 ‰, which reflects the anticipated uncertainty in operationally dependent isotope measurement comparisons between the steroid-specific CO_2 and a VPDB-traceable standard CO_2. Consequently, the total uncertainty of a sample measurement referenced to VPDB, with uncertainties propagated down the traceability chain by root-of-summed-variance, is estimated as 0.12 ‰. Traceability is commonly not extended to the Systeme International (SI) for delta measurements since this extra comparison (Link 1) introduces a large uncertainty that is unimportant in most applications.

RECOMMENDATIONS

The following recommendations are offered to help achieve the goal of international harmonization in GC-C-IRMS performance and results:

1. Identify specific analytical targets and acceptable limits of measurement uncertainty that are based on tolerable risks of false negative and false positive results.
2. Develop a suitable urine RMs, covering the range of observed steroid isotopic values, and assign documented isotopic values and uncertainties that are traceable to VPDB.
3. Develop performance-based standards to assess data quality through intercomparisons and proficiency tests.
4. In comparing two steroid measurements for a significant difference ($\Delta\delta$), these measurements should be performed under identical conditions and referenced to a RM to establish traceability to VPDB. Standard uncertainties of these two measurements can be used in a two-sided normal test (e.g., Natrella, 1963, Section 3-3.1.3) to determine whether the mean results are statistically distinct at a specified confidence level.

REFERENCES

1. Craig, H. (1957) *Geochim. Cosmochim. Acta* **12**, 133-149.
2. Eurochem/CITAC (2000) Guide to Quantifying Uncertainty in Analytical Measurement
3. Hut, G. (1987) Report of IAEA Consultants' group meeting on stable isotope reference samples
4. ISO-BIPM (1993) Guide to the Expression of Uncertainty in Measurement
5. ISO/IEC 17025 (1999) Requirements for the Competence of Calibration and Testing Laboratories
6. ISO-VIM (1993) International Vocabulary of Basic and General Terms in Metrology
7. Natrella, M. G. (1963) Experimental Statistics, NBS Handbook 91 (Reprinted 1966)
8. NIST SP260-149 (2003) Value Assignments and Uncertainty Estimation of Selected Light Stable Isotope Materials

THEVIS
I think it was 2ml up to 20 ml. The average was 10 ml. So if we look up our standard operations procedures, it actually depends on the density and the amount of urine that was actually available. The average is approximately 10ml.

AGUILERA
Ten milliliters of urine is just about 20,000 ng of andro/etio. If you use an HPLC purification, do you know if there were some fractionation? When you do purification by HPLC, it is easy to have isotopic fractionation.

THEVIS
That is the reason why the fractions are pretty broad. As you saw the fractions were not tiny, and you could observe this phenomenon if they were smaller. That is why the fraction is about 2 minutes although the peak width is only 20 seconds, just for example. We need these high amounts because we focus our instrument to maximum linearity, so we sacrifice the sensitivity. So we try to compensate these things.

KAZLAUSKAS
You mentioned that you do a screening first without HPLC. Do you do the HPLC only for confirmation?

THEVIS
That is right.

HOUGHTON
How do you account for variation in the difference between ERC and A/E? You got that slight difference of 1, how do you account for that?

THEVIS
It is a well known phenomenon that you will have this difference. It occurs in basically every sample. So we didn't find that it was only with our reference population but basically every sample that we analyzed. That the E delta values are different by approximately 1 compared to A.

underestimated, by about a factor of ten or more, the variation in measurement results observed across the laboratories. This suggested strongly that variations in the TMP used in each laboratory were responsible for the observed dispersion in results, and that another strategy was needed to enable better reproducibility of results and allow precise and accurate assignments of delta values for the RMs.

The Light Stable Isotope Working Group, an international panel of experts convened by the IAEA, designed an exercise for the isotopes of carbon and oxygen in nine RMs. This exercise was performed by a small number of laboratories, selected on the basis of their experience with carbonate and water sample preparations, and with an attempt to represent equally the variety of IRMS instruments. Standard chemical preparation procedures and sample measurement procedures, determined as "best practices," were mandatory, as were the documentation and reporting of non-mandatory (discretionary) procedures. Additionally, the raw measurement data for all measurements was reported. The measurement data was centrally processed using internationally accepted procedures so that no variations could enter during this stage of the TMP. Lastly, repeatability and consistency of replicated measurements reported in each laboratory formed the basis for analytical performance metrics. These metrics were used to assess the Type A uncertainty of each result relative to the Type B uncertainty estimated by the interlaboratory variation of mean results. When Type A uncertainty of any datum exceeded 0.2 ‰, which was about the upper limit of observed Type B uncertainty for the most challenging RM, that datum was excluded from the mean. The outcome of this exercise was that of 100 results calculated across the laboratories, there was only one 2-s outlier, and that upon inspection of discretionary procedures had an obvious explanation. Intra-laboratory repeatability matched relatively well with inter-laboratory reproducibility, and the value assignments resulting from the exercise were precise, technically sound, and consistent with a consensus mean of globally equitable data.

USADA 0887

GC-C-IRMS Research at UCLA

Don Catlin, M.D.
University of California, Los Angeles

Our group became interested in carbon isotope ratio methodologies when Daniel Fraisse, Ph.D., visited UCLA in 1986. Fraisse described that there is a measurable difference in ^{13}C content between natural vanillin, which is expensive, and synthetic vanillin, which is inexpensive and often sold in place of natural vanillin, and that his institution in France had a GC-C-IRMS facility where such vanillin analyses were done. We wondered if there might also be a measurable difference between pharmaceutical and endogenous urinary **T**. By 1990, Southan et al[1] presented an abstract at a meeting in Barcelona that confirmed this possibility. When Fraisse's collaborators Becchi and Aguilera et al[2] reported that in urine collected before and after **T** administration, the $\delta^{13}C$ ("delta values") of urinary T and metabolites were significantly lower after **T** administration and that the delta values of metabolic precursors of **T** were unchanged, we began to collaborate with them.

Initially Aguilera joined our group but continued to work in France because we did not have an isotope ratio mass spectrometer (IRMS). It took three more years and several papers[2,3,4] to convince sport funding agencies in the U.S. that the method was sound and could ultimately resolve some of the difficulties with interpretation of T/E ratios that exceed 6. In due course we have verified and validated the diol method in our laboratory, and reported delta values in 73 healthy control subjects.[5,7] We believe it is possible to differentiate between urine samples from athletes with the naturally elevated T/E syndrome and athletes who have used **T**.[7] Although the latter manuscript cannot prove the concept, because we do not yet have good ways to define what the syndrome is, it does suggest that eventually this objective might be obtained.

Later we extended the application of the method to **A** and **E**[6] and to epi-T.[8] For T/E cases and for general screening we greatly prefer the Androsterone/**E** assay, even though it does not include an endogenous reference standard, because it is substantially shorter and simpler than the diol assay. Further the low concentrations of diols in some male

urines and most female urines greatly limit the applicability of the diol assay.

The epi-T method is sound and relatively robust. The mean delta value for 43 healthy males controls was -23.8 $^{0}/_{00}$ which is similar to other urinary steroids. We do not have a reference group for females. It is difficult to fully validate the method in man because epi-T is not approved for human use in the U.S. Therefore we cannot administer epi-T unless we obtain approval from the U.S. Food and Drug Administration—an onerous and expensive procedure. Nevertheless we have encountered one subject with a very high urine epi-T level (1,176 ng/mL). The delta value of this person's epi-T (-32.6 $^{0}/_{00}$) was very low as expected. We hope to obtain FDA approval for epi-T studies in the near future.

We have now extended the method to AED. Six male subjects, all of whom denied participation in competitive weightlifting, a history of cardiopulmonary disease, malignancy, prostate disease, major psychiatric disease, substance abuse, or use of any medication known to affect steroid hormone or binding protein levels, were recruited. Subjects were also excluded if they reported prior use of AED or androgenic anabolic steroids. All subjects were required to have normal serum T and creatinine levels, and normal liver function tests. All participants gave written informed consent and all urine was collected for three consecutive days. Days 1 and 3 were control days and no drug was administered. On day 2 the subjects received 50 mg of AED per os and all subsequent urine was collected for the ensuing 24 hours.

USADA 0888

Chemistry (CITAC) (2000) (http://www.measurementuncertainty.org and at http://physics.nist.gov/cuu/Uncertainty).

PRACTICAL ISSUES: A RELEVANT EXAMPLE

Reference materials (RMs) are considered one of the critical components in the assurance of analytical quality, allowing a measure of accuracy within laboratories and an assessment of interlaboratory reproducibility and harmony. For applications important to national and international interests, reliable RMs have been developed and certified by various National Measurement Institutes (NMI) and National Reference Laboratories.

The NIST, as the NMI of the United States, has worked with the IAEA and other organizations to provide a limited number of isotope RMs, mainly pure compounds, for use to test analytical procedures, calibrate instruments, and to enable measurement traceability to internationally accepted reference scales. Value assignments for most of these isotopic RMs were determined through international comparison exercises. The first exercises were "result intercomparisons" where the materials were distributed to all laboratories interested in participating. Those laboratories that responded simply reported a measurement result and uncertainty for each material. In all these exercises, the uncertainty reported from any laboratory grossly

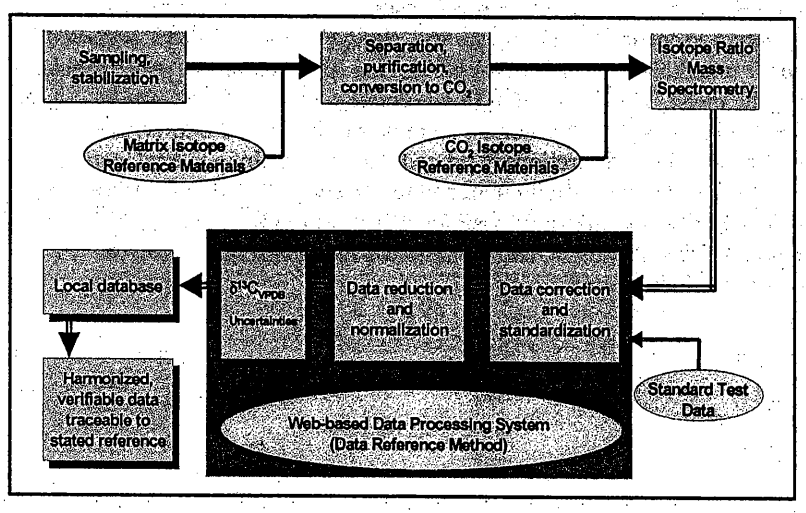


Figure 1. The GC-C-IRMS Total Measurement Process (TMP). A schematic depiction of the flow of chemical and digital information from sampling to reporting of results. The TMP considers every step in the analytical train, and shows the use of quality assurance tools (designated by ovals) that test the fidelity of the analytical steps "downstream" from their input.

underestimated, by about a factor of ten or more, the variation in measurement results observed across the laboratories. This suggested strongly that variations in the TMP used in each laboratory were responsible for the observed dispersion in results, and that another strategy was needed to enable better reproducibility of results and allow precise and accurate assignments of delta values for the RMs.

The Light Stable Isotope Working Group, an international panel of experts convened by the IAEA, designed an exercise for the isotopes of carbon and oxygen in nine RMs. This exercise was performed by a small number of laboratories, selected on the basis of their experience with carbonate and water sample preparations, and with an attempt to represent equally the variety of IRMS instruments. Standard chemical preparation procedures and sample measurement procedures, determined as "best practices," were mandatory, as were the documentation and reporting of non-mandatory (discretionary) procedures. Additionally, the raw measurement data for all measurements was reported. The measurement data was centrally processed using internationally accepted procedures so that no variations could enter during this stage of the TMP. Lastly, repeatability and consistency of replicated measurements reported in each laboratory formed the basis for analytical performance metrics. These metrics were used to assess the Type A uncertainty of each result relative to the Type B uncertainty estimated by the interlaboratory variation of mean results. When Type A uncertainty of any datum exceeded 0.2 ‰, which was about the upper limit of observed Type B uncertainty for the most challenging RM, that datum was excluded from the mean. The outcome of this exercise was that of 100 results calculated across the laboratories, there was only one 2-s outlier, and that upon inspection of discretionary procedures had an obvious explanation. Intra-laboratory repeatability matched relatively well with inter-laboratory reproducibility, and the value assignments resulting from the exercise were precise, technically sound, and consistent with a consensus mean of globally equitable data.

GC-C-IRMS Research at UCLA

Don Catlin, M.D.
University of California, Los Angeles

Our group became interested in carbon isotope ratio methodologies when Daniel Fraisse, Ph.D., visited UCLA in 1986. Fraisse described that there is a measurable difference in ^{13}C content between natural vanillin, which is expensive, and synthetic vanillin, which is inexpensive and often sold in place of natural vanillin, and that his institution in France had a GC-C-IRMS facility where such vanillin analyses were done. We wondered if there might also be a measurable difference between pharmaceutical and endogenous urinary **T**. By 1990, Southan et al[1] presented an abstract at a meeting in Barcelona that confirmed this possibility. When Fraisse's collaborators Becchi and Aguilera et al[2] reported that in urine collected before and after **T** administration, the $\delta^{13}C$ ("delta values") of urinary **T** and metabolites were significantly lower after **T** administration and that the delta values of metabolic precursors of **T** were unchanged, we began to collaborate with them.

Initially Aguilera joined our group but continued to work in France because we did not have an isotope ratio mass spectrometer (IRMS). It took three more years and several papers[2,3,4] to convince sport funding agencies in the U.S. that the method was sound and could ultimately resolve some of the difficulties with interpretation of T/E ratios that exceed 6. In due course we have verified and validated the diol method in our laboratory, and reported delta values in 73 healthy control subjects.[5,7] We believe it is possible to differentiate between urine samples from athletes with the naturally elevated T/E syndrome and athletes who have used **T**.[7] Although the latter manuscript cannot prove the concept, because we do not yet have good ways to define what the syndrome is, it does suggest that eventually this objective might be obtained.

Later we extended the application of the method to **A** and **E**[6] and to epi-T.[8] For T/E cases and for general screening we greatly prefer the Androsterone/**E** assay, even though it does not include an endogenous reference standard, because it is substantially shorter and simpler than the diol assay. Further the low concentrations of diols in some male

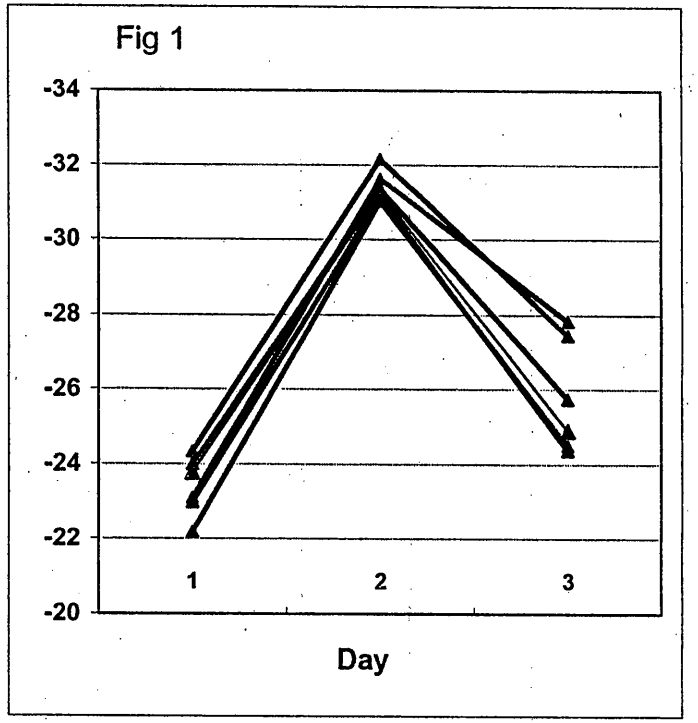
http://www.nist.gov/public_affairs/standards.htm. Of particular interest may be **Laboratory Accreditation Activities in the U.S.** (http://ts.nist.gov/ts/htdocs/210/ ncsci/primer1.htm), the **National Voluntary Laboratory Accreditation Program: Procedures and General Requirements** (http://ts.nist.gov/ts/htdocs/210/214/docs/final-hb150-2001.pdf), and the **International Program of Legal Metrology** (http://ts.nist.gov/ts/htdocs/210/tsag/oiml.htm). Note that U.S. accreditation standards have been harmonized with the International Organization of Standards (ISO)/IEC 17025 (Requirements for the Competence of Calibration and Testing Laboratories).

Traceability. The **International Vocabulary of Basic and General Terms in Metrology (ISO VIM, 2nd ed., 1993, entry 6.10)** states that traceability is a *"property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties."* The National Institute of Standards and Technology (NIST) position on this subject is found at http://www.nist.gov/traceability. For $\delta^{13}C$ measurements, the accepted reference of the traceability chain is VPDB, which may be realized only through comparisons that include the carbonate NBS 19 (NIST/ International Atomic Energy Agency [IAEA] Reference Material 8544). Other comparisons in the chain typically involve a non-carbonate Reference Material (RM) standardized against NBS 19, as well as a laboratory material standardized against the RM.

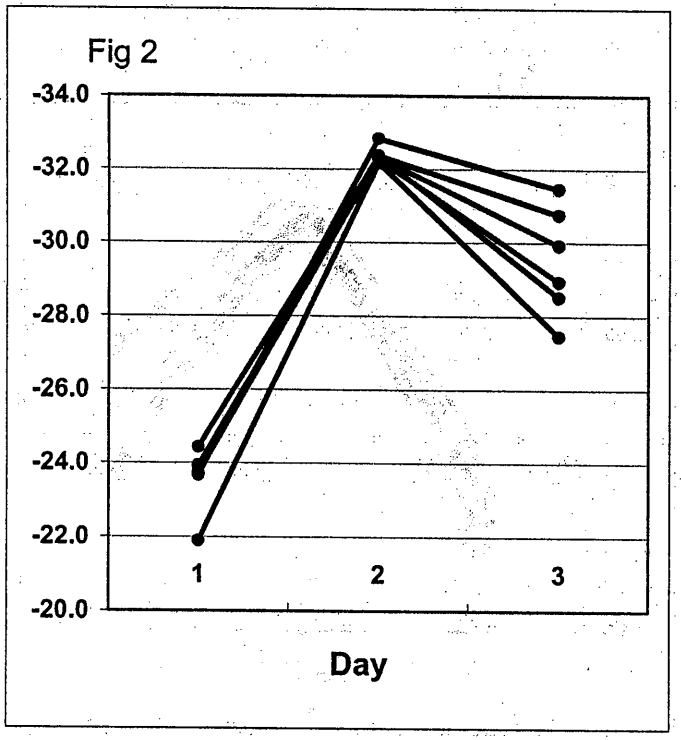
Uncertainty. The **International Vocabulary of Basic and General Terms in Metrology (ISO VIM, 2nd ed., 1993, entry 3.9)** states that uncertainty (of measurement) is a *"parameter, associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand."* Furthermore, uncertainty components are placed in one of two categories, A and B, based on their method of evaluation. Type A standard uncertainty is calculated from a set of repeated observations and is the square root of s^2, the statistically estimated variance. Type B standard uncertainty is obtained from an assumed distribution based on scientific judgment of all possible sources of measurement variability. This is usually based on a pool of comparatively reliable information. The essentials of expressing measurement uncertainty (basic definitions, evaluating uncertainty components, combining uncertainty components, expanded uncertainty, and examples of uncertainty statements may be found in **Guide to the Expression of Uncertainty in Measurement, ISO/ International Bureau of Weights & Measures (BIPM) (1993), Quantifying Uncertainty in Analytical Measurement, Eurachem/ Co-Operation on International Traceability in Analytical**

USADA 0889

Figure 1 shows that on day 1, the baseline day, the delta values for the acetate derivatives of urinary A of the six subjects ranged between -22 and -24 $^0/_{00}$. On day 2 all delta values peaked between -31 and -32 $^0/_{00}$ (close to the maximal theoretical value) for the administered AED. None of the values returned completely to baseline on day 3, but all were becoming less negative indicating that the exogenous substance was being excreted, but still present in some amount.



The pattern for **E** (Fig 2) is similar except that the values recover considerably slower compared to **A**. These findings indicate substantial differences between **A** and **E** in the clearance of exogenous AED, and offer the possibility that such studies represent an interesting new probe of metabolism of endogenous steroids.



USADA 0890

Standardization of Isotope Ratio Measurements for Doping Control

Michael Verkouteren, Ph.D.
NIST

INTRODUCTION

Pharmaceutical steroids are known to differ in isotopic composition from those produced naturally in the body. By measuring the $^{13}C/^{12}C$ ratio of specific steroids detected in urine, the technique of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) can distinguish between exogenous and natural steroids. As a result, the technique has been designated a definitive test of steroid abuse by the World Anti-Doping Association (WADA) and the USADA, and accepted as evidence in adjudication cases by the IOC. While individual laboratories have successfully applied GC-C-IRMS for isotopic measurements of steroids, little is actually known regarding the reproducibility of these measurements across laboratories since standard practices and reliable reference materials do not exist on which to base an assessment. The IOC and USADA have articulated the need to establish a harmonized international network of laboratories, with each laboratory proven to be equivalent in analytical performance. Measurements from such a network would provide sound and globally equitable data needed to support and strengthen decisions made by IOC officials and foster confidence in those decisions by the athlete and world communities.

This extended abstract will outline briefly some of the metrology issues that should be addressed in order to establish equivalence of analytical results across a laboratory network, discuss the lessons learned from prior exercises of isotopic measurement harmonization, and offer some recommendations specific to this GC-C-IRMS application.

METROLOGY ISSUES: TERMS OF REFERENCE

Defined are selected terms of reference that are central to international chemical assay harmonization. Further development of these terms is beyond the scope of this abstract, but their consideration and integration into the analytical results used for doping control is vital for establishing a harmonized network of laboratories.

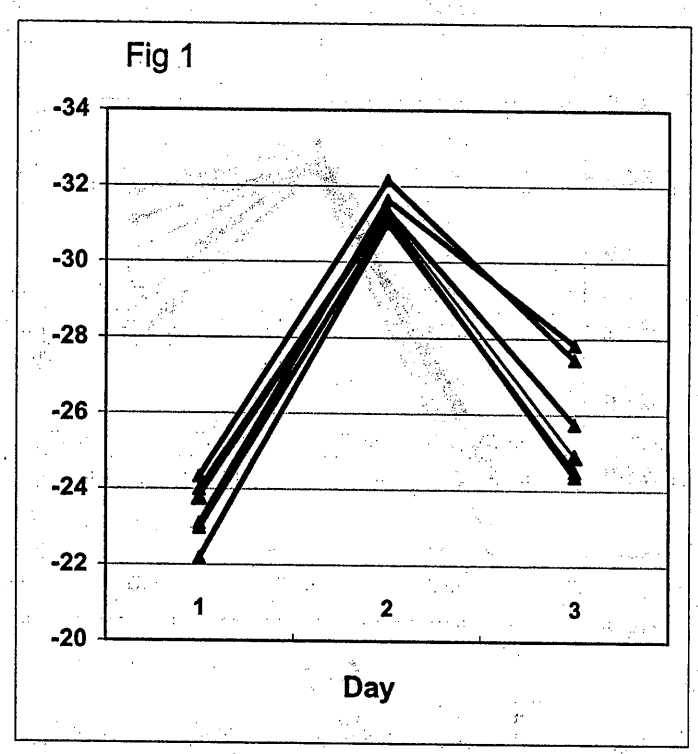
Standards. This term has several meanings in metrology. For detailed information on *measurement standards*, *documentary standards*, and *accreditation standards*, please visit

http://www.nist.gov/public_affairs/standards.htm. Of particular interest may be **Laboratory Accreditation Activities in the U.S.** (http://ts.nist.gov/ts/htdocs/210/ ncsci/primer1.htm), the **National Voluntary Laboratory Accreditation Program: Procedures and General Requirements** (http://ts.nist.gov/ts/htdocs/210/214/docs/final-hb150-2001.pdf), and the **International Program of Legal Metrology** (http://ts.nist.gov/ts/htdocs/210/tsag/oiml.htm). Note that U.S. accreditation standards have been harmonized with the International Organization of Standards (ISO)/IEC 17025 (Requirements for the Competence of Calibration and Testing Laboratories).

Traceability. The **International Vocabulary of Basic and General Terms in Metrology (ISO VIM, 2nd ed., 1993, entry 6.10)** states that traceability is a *"property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties."* The National Institute of Standards and Technology (NIST) position on this subject is found at http://www.nist.gov/traceability. For $\delta^{13}C$ measurements, the accepted reference of the traceability chain is VPDB, which may be realized only through comparisons that include the carbonate NBS 19 (NIST/ International Atomic Energy Agency [IAEA] Reference Material 8544). Other comparisons in the chain typically involve a non-carbonate Reference Material (RM) standardized against NBS 19, as well as a laboratory material standardized against the RM.

Uncertainty. The **International Vocabulary of Basic and General Terms in Metrology (ISO VIM, 2nd ed., 1993, entry 3.9)** states that uncertainty (of measurement) is a *"parameter, associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand."* Furthermore, uncertainty components are placed in one of two categories, A and B, based on their method of evaluation. Type A standard uncertainty is calculated from a set of repeated observations and is the square root of s^2, the statistically estimated variance. Type B standard uncertainty is obtained from an assumed distribution based on scientific judgment of all possible sources of measurement variability. This is usually based on a pool of comparatively reliable information. The essentials of expressing measurement uncertainty (basic definitions, evaluating uncertainty components, combining uncertainty components, expanded uncertainty, and examples of uncertainty statements may be found in **Guide to the Expression of Uncertainty in Measurement, ISO/ International Bureau of Weights & Measures (BIPM) (1993), Quantifying Uncertainty in Analytical Measurement, Eurachem/ Co-Operation on International Traceability in Analytical**

Figure 1 shows that on day 1, the baseline day, the delta values for the acetate derivatives of urinary A of the six subjects ranged between -22 and -24 $^0/_{00}$. On day 2 all delta values peaked between -31 and -32 $^0/_{00}$ (close to the maximal theoretical value) for the administered AED. None of the values returned completely to baseline on day 3, but all were becoming less negative indicating that the exogenous substance was being excreted, but still present in some amount.



Session 3:

Uncertainty in GC-C-IRMS Measurement as Applied to Doping Control

Chair:

Dwight Matthews

USADA 0891

REFERENCES

1. Southan G, Mallet J, Jumeau S, Craig N, Poojara D, Mitchell M et al. Unknown. Programme and Abstracts of the Second International Symposium on Applied Mass Spectrometry in Health Sciences, Barcelona, 1990, Abstract, 1990.

2. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of T in sport. Rapid Commun Mass Spectrom 1994; 8(4):304-308.

3. Shackleton CH, Phillips A, Chang T, Li Y. Confirming T administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids 1997; 62:379-387.

4. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG. Improved method of detection of T abuse by gas chromatography/ combustion/isotope ratio mass spectrometry analysis of urinary steroids. J Mass Spectrometry 1996;31:169-176.

5. Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, Swerdloff RS, Pope HG, Hatton CK. Screening urine for exogenous T by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. J Chromatog B 1999;727:95-105.

6. Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary A and E $\delta^{13}(^{0}/_{00})$ values by gas chromatography/combustion /isotope ratio mass spectrometry. Rapid Comm Mass Spec 2000;14:2294-2299.

7. Aguilera R, Chapman TE, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with T based on urine diols: controls and athletes with elevated T/epi-T ratios. Clin Chem 2001;47:292-300.

8. Aguilera R, Hatton CK, Catlin DH. Detection of doping with epi-T by isotope ratio mass spectrometry. Clin Chem 2002;48:629-636.

Question & Answer Discussion

AUCHUS
Does this Asian pattern run in families? Can you score it as a quantitative trait and then do linkage analysis to find the gene for 17α-HSDs?

CATLIN
I wish I could answer that. We have one person, who happens to be Caucasian, who falls into this pattern. He sends us samples from his sons and daughters. It is not very clear.

AUCHUS
The pattern where you see the 5α Adiol but not the 5β Adiol, couldn't that be explained by someone taking a combination of DHT and T, and shouldn't that be reported as positive?

CATLIN
It could be explained that way and other ways. It is the "shouldn't it be proven as positive" that is the problem. Because as soon as your report it positive you have to prove it. Not only do you have to prove it, you better well have a lot of clinical studies that demonstrate it or you will lose in the CAS.

AUCHUS
Has anyone taken the Chinese samples from the Asian games where the Mitsubishi assays were used for the 5α/5β and gone back and run them with carbon isotope ratio?

BOWERS
We will hear about that in a few minutes from Dr. Ueki.

MATTHEWS
Back to the Asian syndrome and the 15% of Caucasians. You know you are getting the T in because you see the results in the metabolites and in the urine. But simply put, where does the T go? What pathway does it follow through, if it is not appearing in the urine?

USADA 0892

- It may be necessary for a committee to establish one method for GC-C-IRMS analysis
- There may be benefit in having multiple methods and multiple ERCs to make it more difficult to avoid detection.
- $\delta^{13}C$ values obtained from derivatized steroids should be corrected for contributions of the derivatizing agent, possibly by reference compound of known isotopic composition

- Reference ranges for target compounds for individual laboratories for populations with different diets is a concern
 - Athlete may change location or diet according to whether they are training, competing, or resting
 - Laboratory does not know the dietary "history" or geographic origin of the athlete competing

REFERENCES

1. Flenker U, Schänzer W. Expression of differences in isotopic composition. In Recent Advances in Doping Analysis (11), W Schänzer, H Geyer, A Gotzmann, U. Mareck, eds., Sport and Buch Strauß, Köln, 2003. pp 179-182.

Session 3:

Uncertainty in GC-C-IRMS Measurement as Applied to Doping Control

Chair:

Dwight Matthews

REFERENCES

1. Southan G, Mallet J, Jumeau S, Craig N, Poojara D, Mitchell M et al. Unknown. Programme and Abstracts of the Second International Symposium on Applied Mass Spectrometry in Health Sciences, Barcelona, 1990, Abstract, 1990.

2. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of **T** in sport. Rapid Commun Mass Spectrom 1994; 8(4):304-308.

3. Shackleton CH, Phillips A, Chang T, Li Y. Confirming **T** administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids 1997; 62:379-387.

4. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG. Improved method of detection of **T** abuse by gas chromatography/ combustion/isotope ratio mass spectrometry analysis of urinary steroids. J Mass Spectrometry 1996;31:169-176.

5. Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, Swerdloff RS, Pope HG, Hatton CK. Screening urine for exogenous **T** by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. J Chromatog B 1999;727:95-105.

6. Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary **A** and **E** $\delta^{13}(^{0}/_{00})$ values by gas chromatography/combustion /isotope ratio mass spectrometry. Rapid Comm Mass Spec 2000;14:2294-2299.

7. Aguilera R, Chapman TE, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with **T** based on urine diols: controls and athletes with elevated T/epi-T ratios. Clin Chem 2001;47:292-300.

The application of GC-C-IRMS has proceeded in different directions in the eleven (11) WADA/IOC-accredited laboratories that are using the technique. The following elements contribute to observed differences between the laboratories:

- Sample clean-up procedures
- Derivatized (e.g., diacetates) versus underivatized target compounds
- Instrumentation [GV Intruments (formerly MicroMass), PDZ Europa, ThermoFinnigan] and instrumental conditions
- Several target compounds for T and its precursors
 - A and E (average or individual values)
 - 5α- and 5β-Androstan-3α,17β-diol
 - T
- ERC – originally proposed by Shackleton as pregnanediol and pregnanetriol. Others include 11-ketoetiocholanolone and 11-hydroxyetiocholanolone.
- There have been several different approaches to detecting an administration of T or its precursors
 - $\delta^{13}C$ reference range for each compound
 - Difference between the $\delta^{13}C$ values for the target compound and an ERC
 - Ratio of the $\delta^{13}C$ values for the index compound and an ERC
 - Combinations of the above
- Use of internal standard compounds
 - 5α-androstan-3β-ol
 - Octacosane
 - Androstanol
 - 17α-methyltestosterone
 - 17α-methylandrostan-3α,17β-diol
- Selected other target compounds for doping agents other than T and its precursors
 - 5α- and 5β-Androstan-3α,17α-diol (for epi-T)
 - Androst-5-ene-3β,17β-diol (for DHEA)
 - NorAndrosterone for nortestosterone and its precursors in very rare cases where the concentration is sufficiently high to allow application of the GC-C-IRMS technique

The following points were made during the discussion:

- The reporting of differences of delta values as opposed to the ratio of delta values is strongly recommended (in addition, see reference [1])
- Early studies used acetate derivatives to improve the chromatography, but the situation has improved and good chromatographic peak shapes can be obtained with underivatized steroids

USADA 0893

CATLIN
We think we are experts in urine, and I suppose we are experts in urine. When you take T, 99% does not show up as T in urine. We are looking at a very tiny fraction. Most of our assays are just looking at the glucuronide (G) fraction. If you read through the literature, people who have been given infusions of T, way back 40 years ago, give you a good idea of pathways.

It is all changing now; you really can't say where each molecule goes of those 99 that are given intravenously. You know the major pathway. But we are only looking at the 1% that gets into the urine and gets glucuronidated and to some extent sulfated.

MATTHEWS
It seems to me if you could, for example, knock out the ability to form the G then you could get around the T/E ratio test. You could open up a big hole if you naturally didn't make the G.

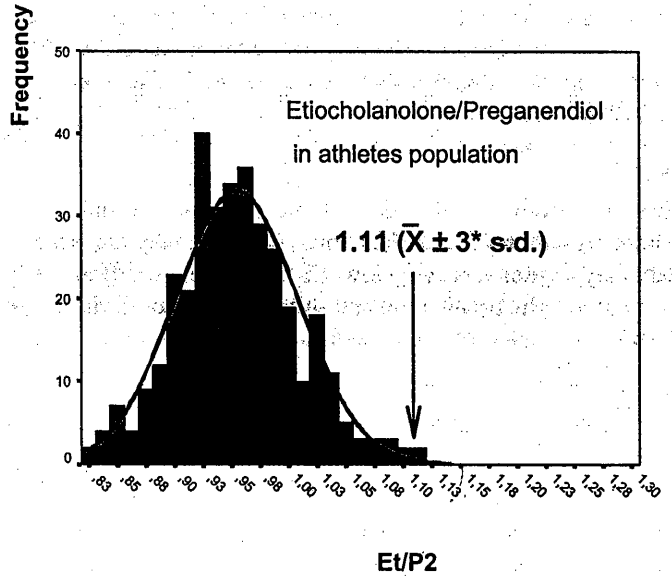
CATLIN
We could have a whole conference on how to beat the test. It may be possible for a serious scientist to develop a way to foil the GC-C-IRMS test. We are trying to do it ourselves because we would like to know before the bad guys do.

BOWERS
Some years ago we did LC-MS-MS to Gs and sulfates of T and epi-T. They are both present in Asian populations. Although they are some what lower, they are not very, very low. Clearly there is a difference in metabolism but not glucuronidation and sulfation; it is somewhere else in the pathway.

GC-C-IRMS for endogenous steroids "Challenging the System"

Jordi Segura
Institut Municipal Investigacio Medica

Natural androgens excreted in human urine, may have a higher content of ^{13}C compared with the synthetic ones (normally derived from soy phytosteroids) and consequently their $^{13}C/^{12}C$ ratio may be different. The detection of a modified $^{13}C/^{12}C$ ratio in urinary **T** or its metabolites could be a direct evidence of the exogenous intake of synthetic **T**, precursors or metabolites. Differences in $^{13}C/^{12}C$ ratio can be determined by carbon isotope ratio mass spectrometry (IRMS). For complex mixtures, this technique can be coupled with a gas chromatograph via a combustion interface (GC-C). Separated organic analytes are combusted to CO_2 and the analysis of the $^{13}CO_2$ and $^{12}CO_2$ molecules allows the calculation of carbon isotope ratios for each compound. These minute differences in carbon isotope ratios are expressed as a per mil deviation (δ ^{13}C ‰) compared to a designated isotopic standard



In this study, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) analysis for the measurement of the δ ^{13}C

USADA 0894

Summary of Discussion:

Basics and Application of GC-C-IRMS in Doping Control

The application of GC-C-IRMS has proceeded in different directions in the eleven (11) WADA/IOC-accredited laboratories that are using the technique. The following elements contribute to observed differences between the laboratories:

- Sample clean-up procedures
- Derivatized (e.g., diacetates) versus underivatized target compounds
- Instrumentation [GV Intruments (formerly MicroMass), PDZ Europa, ThermoFinnigan] and instrumental conditions
- Several target compounds for **T** and its precursors
 - **A** and **E** (average or individual values)
 - 5α- and 5β-Androstan-3α,17β-diol
 - **T**
- ERC – originally proposed by Shackleton as pregnanediol and pregnanetriol. Others include 11-ketoetiocholanolone and 11-hydroxyetiocholanolone.
- There have been several different approaches to detecting an administration of **T** or its precursors
 - $\delta^{13}C$ reference range for each compound
 - Difference between the $\delta^{13}C$ values for the target compound and an ERC
 - Ratio of the $\delta^{13}C$ values for the index compound and an ERC
 - Combinations of the above
- Use of internal standard compounds
 - 5α-androstan-3β-ol
 - Octacosane
 - Androstanol
 - 17α-methyltestosterone
 - 17α-methylandrostan-3α,17β-diol
- Selected other target compounds for doping agents other than **T** and its precursors
 - 5α- and 5β-Androstan-3α,17α-diol (for epi-T)
 - Androst-5-ene-3β,17β-diol (for DHEA)
 - NorAndrosterone for nortestosterone and its precursors in very rare cases where the concentration is sufficiently high to allow application of the GC-C-IRMS technique

The following points were made during the discussion:

- The reporting of differences of delta values as opposed to the ratio of delta values is strongly recommended (in addition, see reference [1])
- Early studies used acetate derivatives to improve the chromatography, but the situation has improved and good chromatographic peak shapes can be obtained with underivatized steroids

CATLIN
We think we are experts in urine, and I suppose we are experts in urine. When you take **T**, 99% does not show up as **T** in urine. We are looking at a very tiny fraction. Most of our assays are just looking at the glucuronide (**G**) fraction. If you read through the literature, people who have been given infusions of **T**, way back 40 years ago, give you a good idea of pathways.

It is all changing now; you really can't say where each molecule goes of those 99 that are given intravenously. You know the major pathway. But we are only looking at the 1% that gets into the urine and gets glucuronidated and to some extent sulfated.

MATTHEWS
It seems to me if you could, for example, knock out the ability to form the **G** then you could get around the T/E ratio test. You could open up a big hole if you naturally didn't make the **G**.

CATLIN
We could have a whole conference on how to beat the test. It may be possible for a serious scientist to develop a way to foil the GC-C-IRMS test. We are trying to do it ourselves because we would like to know before the bad guys do.

BOWERS
Some years ago we did LC-MS-MS to **G**s and sulfates of **T** and epi-**T**. They are both present in Asian populations. Although they are some what lower, they are not very, very low. Clearly there is a difference in metabolism but not glucuronidation and sulfation, it is somewhere else in the pathway.

In conclusion, the GC-C-IRMS usefulness applied to natural steroid control in breeding animals has been demonstrated. Some improvements are still necessary to make the technique more efficient (instrumental robustness, sensitivity, validation, criteria of positivity...) and finally applicable to matrices more challenging in terms of residue levels.

Question & Answer Discussion

BRENNA
PTV injection, what does stand for?

LI BIZEC
Large volume injector (Programmed Temperature Volatilization)

HALE
You were using this with cattle. There are a lot of differences in cattle, bulls, females, and most importantly the neutered cattle. Did you make any different change in account for that? Second question, you showed some very interesting slides on **T**, but you also said you used estrAdiol. But I did not see any extra slides on estrAdiol, and how that had an impact on the cattle and steroidogenesis.

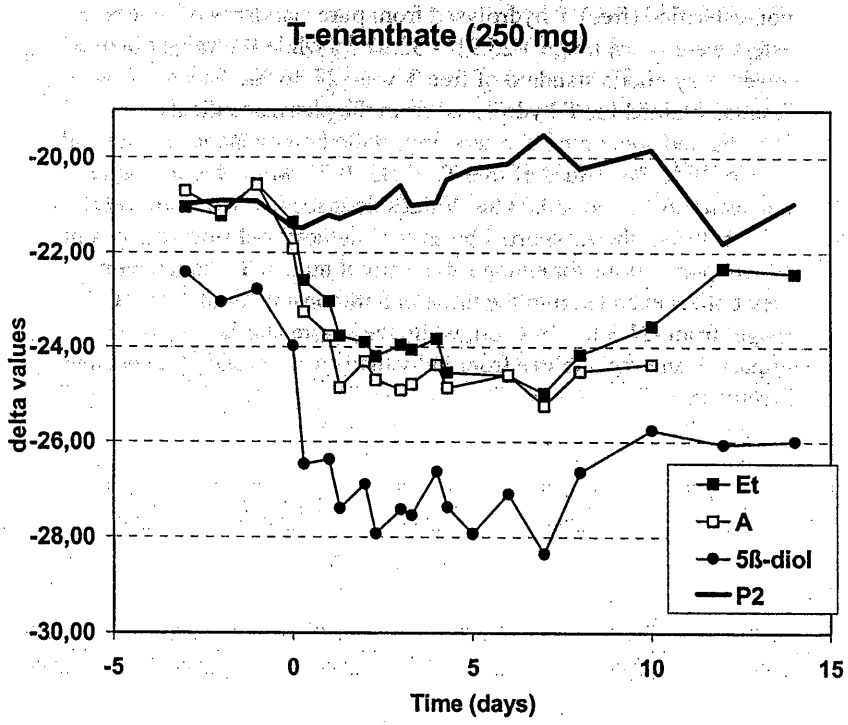
LE BIZEC
What we are doing in Austria with the project ISOSTER, It is not protected, but all of the results must be agreed upon before verification. Can you explain the first question?

HALE
In the US, we take a male cow and take off the testes. Was that type of animal being studied or were you studying animals with in-tact gonads. Castrated versus not castrated animals?

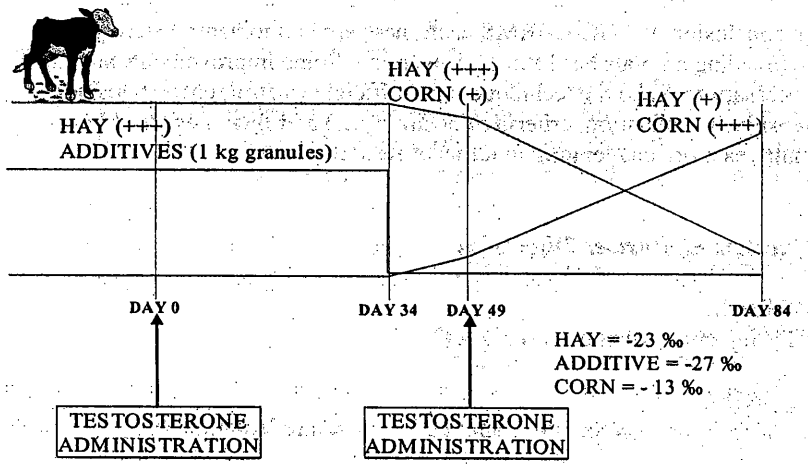
LE BIZEC
In general what is practiced in farms is that I always compare to the opposite gender. So I used castrated males and females. I have been presented with web data that explains that there is no real difference. There is not a significant difference between castrated males and females.

USADA 0895

‰ value of **T** obtained from esterified forms of pharmaceutical preparations and pure standards obtained world-wide was investigated after applying different hydrolytic procedures. Mean $\delta\ ^{13}C$ ‰ values of non-esterified (free) **T** hydrolysed from pure standards of several **T** esters were in the range: –25.91/ - 32.82 ‰ while the value obtained for a (semi)-synthetic standard of free **T** was -27.36 ‰. The $\delta\ ^{13}C$ ‰ results obtained for **T** hydrolysed from the pharmaceuticals investigated containing **T** esters were quite homogeneous (mean and S.D. of $\delta\ ^{13}C$ ‰ values of free **T**: 27.43±0.76 ‰), being the range between -26.18 and -30.04 ‰. Values described above were clearly different from those reported by several authors and studied by us in athlete populations for endogenous natural human **T** and its main metabolites excreted into the urine in non-consumers of **T** ($\delta\ ^{13}C$ ‰ range: from -21.3 to -24.4 ‰), while they were similar to those of urinary **T** and metabolites from individuals treated with **T** esters and **T** precursors.



USADA 0896



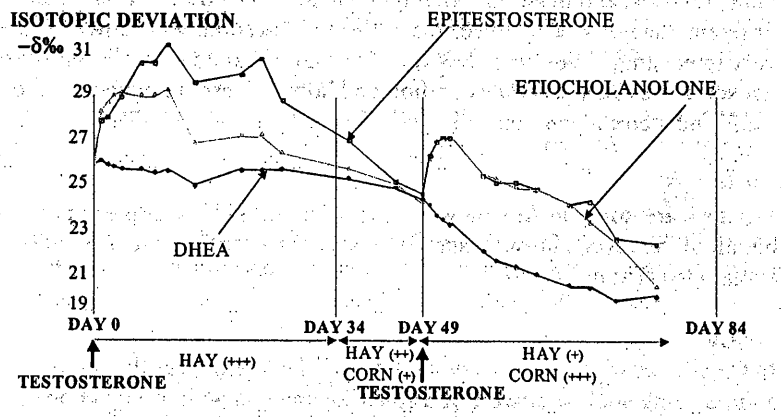


Figure 2: Evaluation of dietary changes over the CIR of ERC and metabolites (above: protocol of the experimentation; underneath: isotopic deviation versus time).

In conclusion, the GC-C-IRMS usefulness applied to natural steroid control in breeding animals has been demonstrated. Some improvements are still necessary to make the technique more efficient (instrumental robustness, sensitivity, validation, criteria of positivity…) and finally applicable to matrices more challenging in terms of residue levels.

Question & Answer Discussion

BRENNA
PTV injection, what does stand for?

LI BIZEC
Large volume injector (Programmed Temperature Volatilization)

HALE
You were using this with cattle. There are a lot of differences in cattle, bulls, females, and most importantly the neutered cattle. Did you make any different change in account for that? Second question, you showed some very interesting slides on T, but you also said you used estrAdiol. But I did not see any extra slides on estrAdiol, and how that had an impact on the cattle and steroidogenesis.

LE BIZEC
What we are doing in Austria with the project ISOSTER, It is not protected, but all of the results must be agreed upon before verification. Can you explain the first question?

HALE
In the US, we take a male cow and take off the testes. Was that type of animal being studied or were you studying animals with in-tact gonads. Castrated versus not castrated animals?

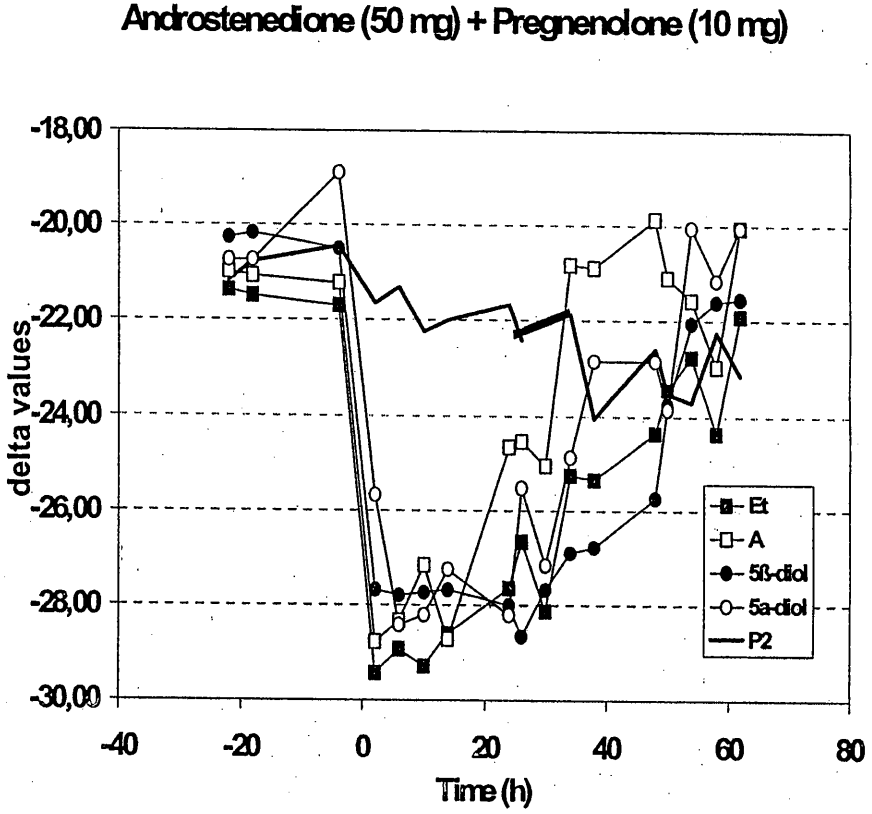
LE BIZEC
In general what is practiced in farms is that I always compare to the opposite gender. So I used castrated males and females. I have been presented with web data that explains that there is no real difference. There is not a significant difference between castrated males and females.

‰ value of T obtained from esterified forms of pharmaceutical preparations and pure standards obtained world-wide was investigated after applying different hydrolytic procedures. Mean $\delta\ ^{13}C$ ‰ values of non-esterified (free) T hydrolysed from pure standards of several T esters were in the range: –25.91/ - 32.82 ‰ while the value obtained for a (semi)-synthetic standard of free T was -27.36 ‰. The $\delta\ ^{13}C$ ‰ results obtained for T hydrolysed from the pharmaceuticals investigated containing T esters were quite homogeneous (mean and S.D. of $\delta\ ^{13}C$ ‰ values of free T: 27.43±0.76 ‰), being the range between -26.18 and -30.04 ‰. Values described above were clearly different from those reported by several authors and studied by us in athlete populations for endogenous natural human T and its main metabolites excreted into the urine in non-consumers of T ($\delta\ ^{13}C$ ‰ range: from -21.3 to -24.4 ‰), while they were similar to those of urinary T and metabolites from individuals treated with T esters and T precursors.

improvable. The first idea was to study the classical silylating mixture MSTFA/TMIS/ dithiothreitol (DTT). Nevertheless, poor $^{13}C/^{12}C$ repeatability was observed and attributed to the catalyst which was presumed to compete with MSTFA during the silylation. The replacement of TMIS with NH_4I led us to determine a new reagent: $MSTFA/NH_4I/DTT/CH_3CN$ (1000:5:0.25:100, v/w/w/v). Inter-day reproducibility of derivatized target steroids on standard was judged acceptable (< ± 0.5‰); peak width was improved and IRMS signal intensities (m/z 44) was in the 5.10^{-9} A order for 20 ng injected. Combustion furnace life is not significantly affected (± 6 months) especially when the effluent gas is vented before entering the combustion furnace. Extracts are injected into the gas chromatograph through a split/splitless injector, and separated onto a capillary column (Macherey-Nagel, δ-3, 30 m 0.25 mm x 0.25 µm).

In bovine urine, two ERCs, four T metabolites, and two estrAdiol metabolites are excreted in sufficiently high amount to be measurable in most cases by GC-C-IRMS. We demonstrated the ability of the method to differentiate ERC and at least two T metabolites on the basis of their $^{13}C/^{12}C$ over a period of 2-3 weeks in bovine urine (whatever breed and age) after IM injection of T esters (250 mg single dose). At this level, we decided to evaluate the consequences of dietary modification on the isotopic deviations of ERC and metabolites. For that (Figure 2), a cow (Normande, 15 years) received a diet based on hay and granules (1 kg daily) during several weeks (period until Day 34), corn replaced granules (Day 34), then corn proportion was slightly augmented (Day 34 to Day 49) and finally considerably increased into the feeding (Day 40 to Day 84). T enanthate (250 mg) was IM injected at Day 0 and Day 49. EA-IRMS of hay, granules and corn gave –23‰, -27‰ and –13‰, respectively. Results clearly showed on the first period (until Day 34) that the ERC remained constant (-25‰). The ^{13}C composition of the two metabolites obviously decreased after T administration and remained significantly different from the ERC over several weeks. The slight diet modification (Day 34 to Day 49, maize introduction) generated a perceptible ^{13}C enrichment of DHEA (± 1‰). Then the massive introduction of corn into the diet noticeably enriched the ^{13}C composition of DHEA. The T injection (Day 49) produced a clear $^{13}C/^{12}C$ modification of both metabolites (maximum (± 4‰) in-between metabolites and precursors). The misuse detectability remained the same in the two phases of the experiment.

USADA 0897



A comprehensive analytical method using GC-C-IRMS allowing the detection of these compounds overcoming the inter-individual variability in steroid profile excretion or metabolism in routine conditions for sports drug testing has been developed. The method proved to be able to detect controlled administrations by different routes of **T**, dihydrotestosterone (DHT), AED, and dehydroepiAndrosterone (DHEA) in healthy volunteers for longer periods than using official criteria, when existing. The obtained data were compared with reference values obtained by healthy non-steroid abusers. Even with the concomitant administration of pregnenolone, masking the normal isotopic content of the natural marker preganendiol, the method is able to clearly demonstrate the ingestion of **T** precursors.

In the absence of blood markers of **T** abuse (testosterone esters, **G** of testosterone metabolites), GC-C-IRMS appears as one of the major tools to detect doping by **T** or **T** precursors.

Question & Answer Discussion

MATTHEWS
From the perspective of methodologies such as GC/MS where we are used to thinking of the wider linear range of quantization than we are with GC-C-IRMS, I was impressed that you had selected a wide range for your isotope ratio measurements. My experience with GC-C-IRMS is that you have a much more limited range in which you can work in for precise ^{13}C enrichment measurements. The $\delta^{13}C$ values that you put up, as a function of concentration, actually were quite good. You had a good range of quantification. What do you think?

SEGURA
Yes, we were not so upset by the range; we obtained the range that is wider than may be expected. We were really disappointed by the low extreme of the range, and we expected we could reach the stability of delta values with lower concentration. We injected less amounts than we in fact needed.

USADA 0898

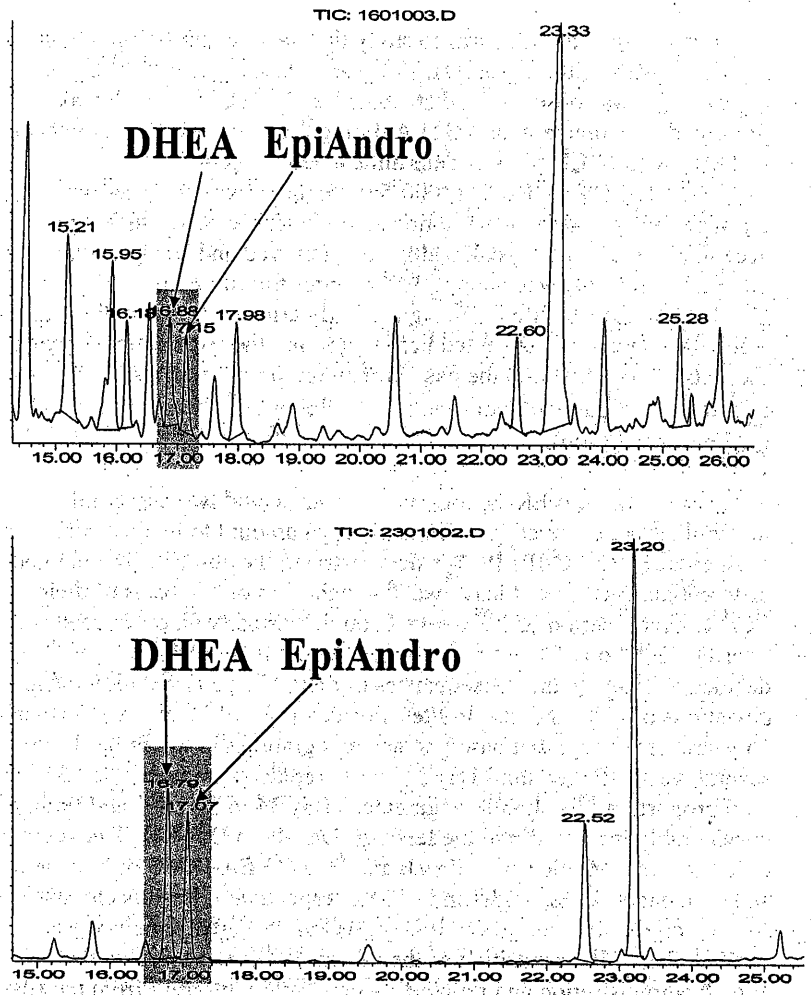
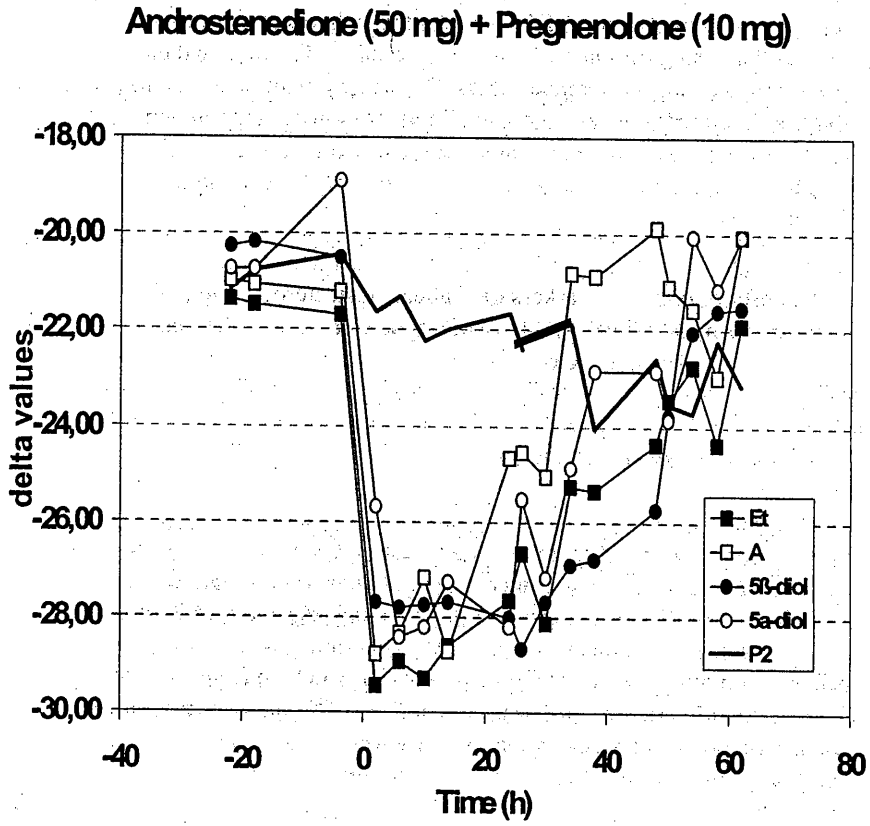


Figure 1: Purification efficiency of a semi-preparative HPLC (aminopropyle stationary phase) prior to any GC-C-IRMS measurement.

On one hand, acetylation derivatization is widely used for steroid analysis by GC-C-IRMS because it leads to good chromatographic behaviour (compared to non-derivatized steroid) without affecting the carbon isotope ratio of the target analyte (two carbon atoms per derivatized site). The analysis of hundreds of samples led us to occasionally observe incomplete derivatization (mixture of mono- and di-acetylated steroids), and the chromatographic behaviour of the derivatized steroids was judged

improvable. The first idea was to study the classical silylating mixture MSTFA/TMIS/ dithiothreitol (DTT). Nevertheless, poor $^{13}C/^{12}C$ repeatability was observed and attributed to the catalyst which was presumed to compete with MSTFA during the silylation. The replacement of TMIS with NH_4I led us to determine a new reagent: MSTFA/NH_4I/DTT/CH_3CN (1000:5:0.25:100, v/w/w/v). Inter-day reproducibility of derivatized target steroids on standard was judged acceptable (< ± 0.5‰); peak width was improved and IRMS signal intensities (m/z 44) was in the 5.10^{-9} A order for 20 ng injected. Combustion furnace life is not significantly affected (± 6 months) especially when the effluent gas is vented before entering the combustion furnace. Extracts are injected into the gas chromatograph through a split/splitless injector, and separated onto a capillary column (Macherey-Nagel, δ-3, 30 m 0.25 mm x 0.25 µm).

In bovine urine, two ERCs, four T metabolites, and two estrAdiol metabolites are excreted in sufficiently high amount to be measurable in most cases by GC-C-IRMS. We demonstrated the ability of the method to differentiate ERC and at least two T metabolites on the basis of their $^{13}C/^{12}C$ over a period of 2-3 weeks in bovine urine (whatever breed and age) after IM injection of T esters (250 mg single dose). At this level, we decided to evaluate the consequences of dietary modification on the isotopic deviations of ERC and metabolites. For that (Figure 2), a cow (Normande, 15 years) received a diet based on hay and granules (1 kg daily) during several weeks (period until Day 34), corn replaced granules (Day 34), then corn proportion was slightly augmented (Day 34 to Day 49) and finally considerably increased into the feeding (Day 40 to Day 84). T enanthate (250 mg) was IM injected at Day 0 and Day 49. EA-IRMS of hay, granules and corn gave –23‰, -27‰ and –13‰, respectively. Results clearly showed on the first period (until Day 34) that the ERC remained constant (-25‰). The ^{13}C composition of the two metabolites obviously decreased after T administration and remained significantly different from the ERC over several weeks. The slight diet modification (Day 34 to Day 49, maize introduction) generated a perceptible ^{13}C enrichment of DHEA (± 1‰). Then the massive introduction of corn into the diet noticeably enriched the ^{13}C composition of DHEA. The T injection (Day 49) produced a clear $^{13}C/^{12}C$ modification of both metabolites (maximum (± 4‰) in-between metabolites and precursors). The misuse detectability remained the same in the two phases of the experiment.



GC-C-IRMS to Control the Misuse of Natural Occurring Steroid in Breeding Animals

Bruno Le Bizec, Corinne Buisson, Véronique Ferchaud, Stéphanie Prevost, Tatiana Nicol, Marie-Aude Kerautret, Bruno Veyrand, Fabrice Monteau and François Andre
French Reference Laboratory

The use of anabolic growth promoting preparations for improving the growth and feed conversion rates of food producing animals has been banned within the European Union since 1988[1]. Whereas the use of strictly xenobiotic steroids decreased since the end of the 90's, the illegal application of natural steroid hormones remains a current practice probably because the classical analytical methodologies developed up till now and applied daily in the control laboratories are still not rigorous enough for this purpose. Because current approaches involve the determination of the absolute concentration, in most cases the results cause controversy. Indeed, no real and validated physiological levels are officially recognised. Other attempts based on hair analysis and demonstration of the steroid esters residues are currently used, but this strategy excludes some steroids (progesterone, norandrostenedione) and becomes non applicable when non-esterified natural occurring steroids are administered.

The most promising and definitive approach remains the determination of the isotopic $^{13}C/^{12}C$ composition of steroids, and the examination of any significant variation in-between ERCs and monitored target metabolites. To be unambiguously applicable to the control, the technique requires a lot of precautions from the sample preparation (extraction, purification and derivatization), to the final IRMS measurement (injection, chromatographic separation and $^{13}C/^{12}C$ determination). Biological samples collected from breeding animals (mainly bovine urine) are particularly complex. The analytical strategy developed in our laboratory since several years includes necessarily at least 2 SPE steps based on complementary chemical principles (C_{18}, SiOH), and a final semi-preparative HPLC (NH_2). This step authorizes drastic purification of the extract (figure 1); nevertheless, there is a clear associated fractionation risk. Even whether the fraction collection should be enlarged to avoid any $^{13}C/^{12}C$ discrimination, the introduction of one internal standard in each collected fraction prevents any troubles.

USADA 0899

MATTHEWS
We are trying to measure a carbon isotope, where the isotope is 100-fold lower than what we normally think of, which is the m/z 45 beam. So when we get down toward the low end, the m/z 45 beam is 100 times lower than the m/z 44, and it is really, really low. I think we are doing very well with a 100-fold linear range. So from my experience, I think what you show is not surprising at all. I think it is excellent that the method is working as well as you have been able to make it work.

Improved detection of T cascades by monitoring delta-parameters of A, etiocholanolone, 5α- and 5β-androstandiols and DHEA

Makoto Ueki, Ph.D.
Mitsubishi Kagaku Bio-Clinical Laboratories

A typical characteristic of steroid profiles of East Asians such as Japanese, Chinese and Korean is extremely low T/E ratio and poor response of their system to administration of **T**. Such difference makes tests for doping with naturally occurring steroids difficult. Ethnic differences in metabolic patterns of precursor steroids have also been observed. Carbon isotope ratio measurement in doping control is particularly useful to differentiate administered synthetic steroids from these with human origin, and doping with **T** precursors can be definitively detected by monitoring delta-parameters of some gateway steroids such as **A**, **E**, 5α- and 5β-androstan-3α,17-β-diols(5aA2, 5bA2), and DHEA.

Pregnenolone is not a fully formed steroid hormone, and its abuse as a source of anabolic steroid is unlikely; however, administration of pregnenolone results in excretion of pregnanediol and –triol; suggesting it can be abused as a masking agent to alter delta- values of ERCs. Our results suggest that normal steroid profiling alone does not constitute scientific evidence of pregnenolone doping, but reduced ^{13}C-content of pregnanediol (P2) or pregnanetriol (P3) relative to that of any of androgenic steroid could be of the sign of its doping. Carbon isotope ratio testing is sufficiently conclusive even if it is a single test. A second carbon isotope ratio test with few days interval may also be useful to follow a suspicious case of pro-hormones abuse. This is useful because a major part of orally administered steroids are eliminated from the body in several days, but intra-individual variation of carbon isotope ratio in human steroids is very small. An acute change of their delta parameters would indicate doping with **T** precursors.

USADA 0900

with what you eat and its relative carbon source. It all gets eventually down to the carbon, liver, and the TCA cycle, makes into what we need to make glucose and fat, and there you go.

When you do these kinds of studies, you could do them very simply. You can take breath samples of a whole variety of populations. If you believe you are what you eat, then the same differences should be seen in breath CO_2. There a quite a few older studies that shows the differences between Asians, Europeans, and Americans. You can tell a lot about what the diet of the person is just from the breath CO_2.

GC-C-IRMS to Control the Misuse of Natural Occurring Steroid in Breeding Animals

Bruno Le Bizec, Corinne Buisson, Véronique Ferchaud, Stéphanie Prevost, Tatiana Nicol, Marie-Aude Kerautret, Bruno Veyrand, Fabrice Monteau and François Andre
French Reference Laboratory

The use of anabolic growth promoting preparations for improving the growth and feed conversion rates of food producing animals has been banned within the European Union since 1988[1]. Whereas the use of strictly xenobiotic steroids decreased since the end of the 90's, the illegal application of natural steroid hormones remains a current practice probably because the classical analytical methodologies developed up till now and applied daily in the control laboratories are still not rigorous enough for this purpose. Because current approaches involve the determination of the absolute concentration, in most cases the results cause controversy. Indeed, no real and validated physiological levels are officially recognised. Other attempts based on hair analysis and demonstration of the steroid esters residues are currently used, but this strategy excludes some steroids (progesterone, norandrostenedione) and becomes non applicable when non-esterified natural occurring steroids are administered.

The most promising and definitive approach remains the determination of the isotopic $^{13}C/^{12}C$ composition of steroids, and the examination of any significant variation in-between ERCs and monitored target metabolites. To be unambiguously applicable to the control, the technique requires a lot of precautions from the sample preparation (extraction, purification and derivatization), to the final IRMS measurement (injection, chromatographic separation and $^{13}C/^{12}C$ determination). Biological samples collected from breeding animals (mainly bovine urine) are particularly complex. The analytical strategy developed in our laboratory since several years includes necessarily at least 2 SPE steps based on complementary chemical principles (C_{18}, SiOH), and a final semi-preparative HPLC (NH_2). This step authorizes drastic purification of the extract (figure 1); nevertheless, there is a clear associated fractionation risk. Even whether the fraction collection should be enlarged to avoid any $^{13}C/^{12}C$ discrimination, the introduction of one internal standard in each collected fraction prevents any troubles.

MATTHEWS
We are trying to measure a carbon isotope, where the isotope is 100-fold lower than what we normally think of, which is the m/z 45 beam. So when we get down toward the low end, the m/z 45 beam is 100 times lower than the m/z 44, and it is really, really low. I think we are doing very well with a 100-fold linear range. So from my experience, I think what you show is not surprising at all. I think it is excellent that the method is working as well as you have been able to make it work.

AHRENS
Actually, the diols were measured for the individuals. The problem with the diol measurements were that there were several instances where concentrations of the diols were too low to get to what we would be confident that the diols were accurate measurement of delta values.

UEKI
I have been following my own delta value for the past five years. The difference was within one unit, only. I have started, three months ago, a high soy diet, and even after losing 14 kilograms of body weight, the delta value does not change at all. My explanation is that the carbon source of steroids does not come from soy. A small amount of meat, cholesterol is enough to generate. This is my understanding.

AUCHUS
Were you referring to the ^{13}C of the diols or were you talking about the ^{13}C of the total plant?

AHRENS
I believe it was the ^{13}C content of the plant, not the specifically of the sterols.

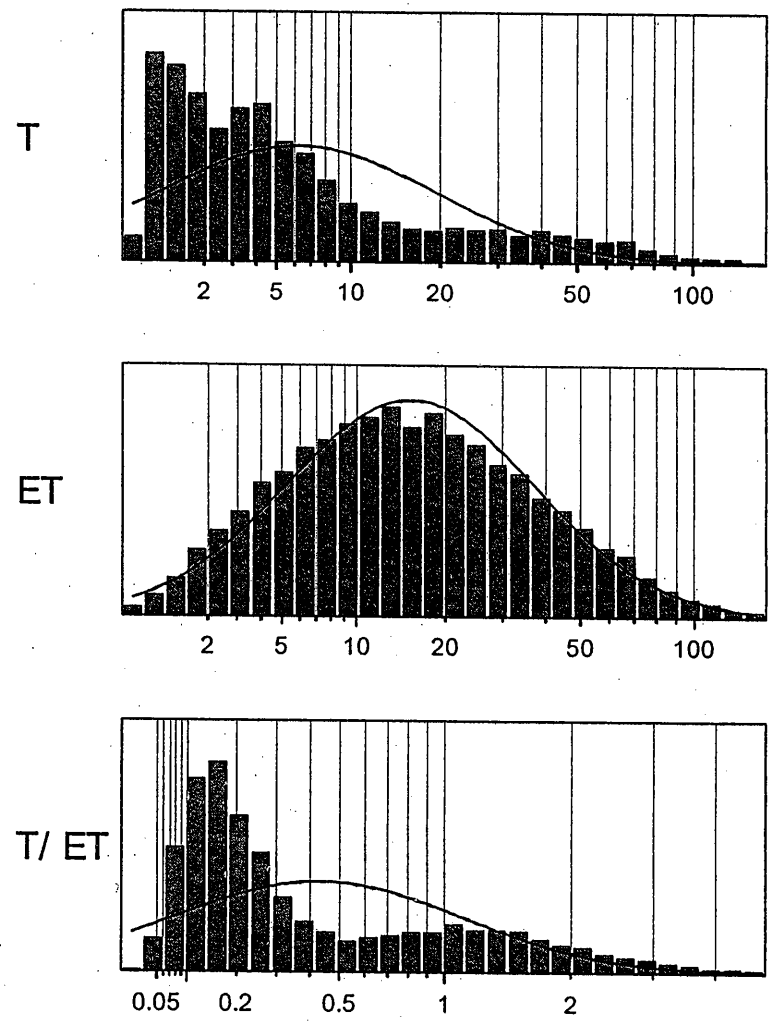
AUCHUS
What is the acetate? The acetate is going into the cholesterol that you are making. So again, the synthetic steroids are reflecting the sterol content unit which is not necessarily what is going into your body and then going into your cholesterol and then your sterol. I think that is the correct explanation, basically.

MATTHEWS
You are right about the acetate. You are what you eat. Same thing for these plants. They are what they fix. They are isotopiclly light or heavy all the way through. So their acetate is isotopically light or heavy all the way through. The acetate may be a little lighter relatively to glucose, for example in these plants, but the overall plant is either isotopiclly light or heavy.

This brings us back to the issue of corn. Because, I would suggest that if an Asian person who is not on a corn diet to begin with shifts his protein source around a little bit going to soy, there would almost be no change in the delta value of his steroids. Because it is probably the corn which is the most important aspect. It has nothing to do with the meat. It just has to do

USADA 0901

Figure-1 Distributions of T parameters in international sports
(n=12,388: ng/ml)



Figures 2, 3, 4; Closed lines correspond to delta-values after administration of synthetic steroids. Dotted lines show delta values of basal level. Experiments were done with 3 each of females and males. Data from the typical case are presented.

Abbreviations:
Suffices "T", "S" and "G" means steroid fractions of total, sulfate and G respectively. Significant differences of delta-parameter of steroids of interest between fractions were not observed



USADA 0902

In summary, farm subjects who subsist on an all-soy diet have more negative A and E $\delta^{13}C$ values compared to a controls. Further studies of diet and populations are planned.

REFERENCES

1. Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary A and E $\delta^{13}(^{0}/_{00})$ values by gas chromatography/combustion /isotope ratio mass spectrometry. Rapid Comm Mass Spec 2000;14:2294-2299.

Question & Answer Discussion

MATTHEWS
So when the dietician interviewed these people, how much corn did they use? They probably would not be eating that much sweet corn, especially in the winter. So it would be corn meal only, so they should be relatively corn free since they are not eating meat that is coming from a corn fed diet.

AHRENS
This is true. We do have the interviews from the dietician. Our target at that point, when the study was set up; we weren't focusing on corn at all. Our thought was basically soy. Most of the questions that were asked of the study subjects were, "How much soy do you take in, in your daily diet?" Every item that the individual would eat that contained any amount of soy was than further analyzed to determine kilocalories of soy material. I cannot tell you right now how much corn was taken in by either the control or the vegan population.

SEGURA
How do these people in high soy diets respond to an administration of T? If they go out of the population range and especially an internal marker would help in the differentiation. Have you planned to do some administration of this personal population?

AHRENS
I would love to be able to do that. I cannot say that there are any plans to undertake that study.

BOWERS
You chose to use the A/E, is there a reason why you choose to do that? Why not in addition to the diols, if you had the option to do that?

AHRENS
Actually, the diols were measured for the individuals. The problem with the diol measurements were that there were several instances where concentrations of the diols were too low to get to what we would be confident that the diols were accurate measurement of delta values.

UEKI
I have been following my own delta value for the past five years. The difference was within one unit, only. I have started, three months ago, a high soy diet, and even after losing 14 kilograms of body weight, the delta value does not change at all. My explanation is that the carbon source of steroids does not come from soy. A small amount of meat, cholesterol is enough to generate. This is my understanding.

AUCHUS
Were you referring to the ^{13}C of the diols or were you talking about the ^{13}C of the total plant?

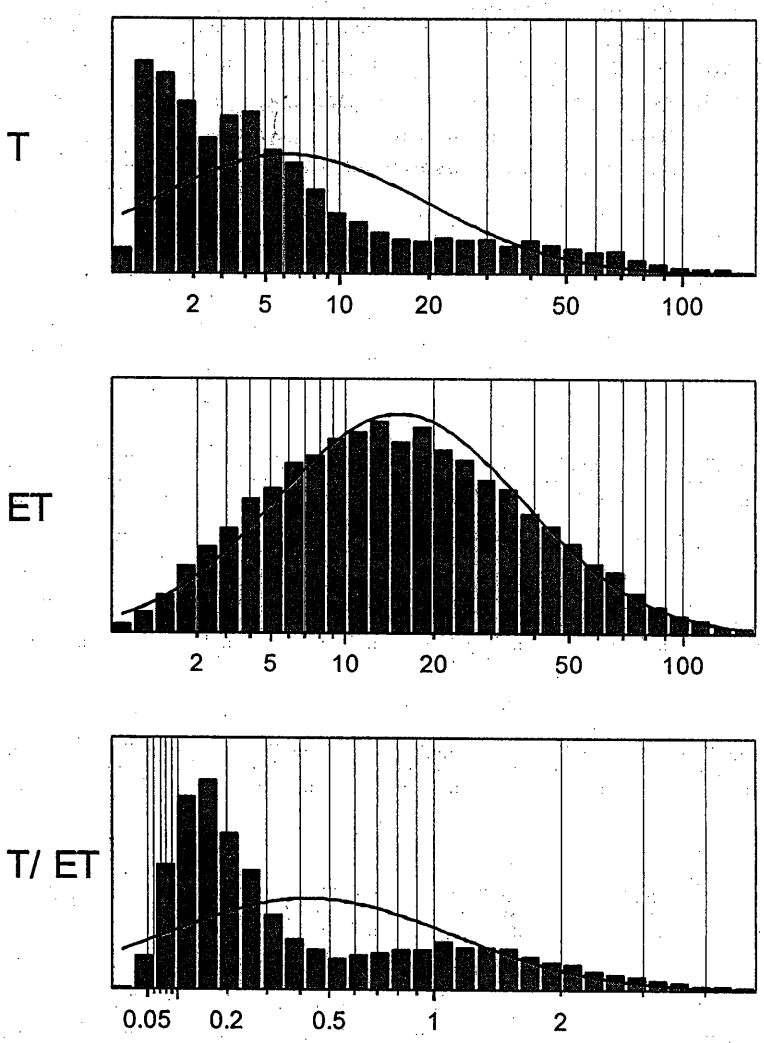
AHRENS
I believe it was the ^{13}C content of the plant, not the specifically of the sterols.

AUCHUS
What is the acetate? The acetate is going into the cholesterol that you are making. So again, the synthetic steroids are reflecting the sterol content unit which is not necessarily what is going into your body and then going into your cholesterol and then your sterol. I think that is the correct explanation, basically.

MATTHEWS
You are right about the acetate. You are what you eat. Same thing for these plants. They are what they fix. They are isotopiclly light or heavy all the way through. So their acetate is isotopically light or heavy all the way through. The acetate may be a little lighter relatively to glucose, for example in these plants, but the overall plant is either isotopiclly light or heavy.

This brings us back to the issue of corn. Because, I would suggest that if an Asian person who is not on a corn diet to begin with shifts his protein source around a little bit going to soy, there would almost be no change in the delta value of his steroids. Because it is probably the corn which is the most important aspect. It has nothing to do with the meat. It just has to do

Figure-1 Distributions of T parameters in international sports
(n=12,388: ng/ml)



Effect of High Soy Diet on GC-C-IRMS Results

Brian Ahrens
University of California, Los Angeles

Plants may be divided into two main types and several minor types. A C_3 plant takes up CO_2 and incorporates the C atoms into 3-phosphoglycerate or 3-Phosphoglyceric Acid (3-PGA). This is the so-called Calvin cycle of C_3 plants, of which sugar beet is an example. Glucose derived from C_3 plants has a $\delta^{13}C$ of about –25 $^{o}/_{oo}$ or lower. In contrast, when C_4 plants fix CO_2 the fixation yields a C_4-dicarboxylic acid (oxaloacetate). This process is also referred to as the Hatch-Slack cycle. The δ values found in C_4 plants range from about –8 to –20 $^{0}/_{00}$ (heavier than C_3 plants because they contain more ^{13}C).

Starting materials for the synthesis of **T** are derived from the soy plant. Since soy plants are C_3 plants that are depleted in ^{13}C, we hypothesized that individuals who ingest soy diets will excrete urinary steroids with low ^{13}C values. To test the hypothesis we conducted a cross-sectional study consisting of 18 control subjects and 23 subjects living on a collective farm whose members were purported to subsist solely on soy products. The farm was located in Summertown, TN and the clinical aspects of the study were conducted at the farm and in the neighboring city of Knoxville, TN.

Compliance with the high soy diet was monitored by quantitating soy isoflavones, namely daidzein and genistein, in the urine of the subjects with an LC-MS-MS assay. The delta values of the acetates of **A** and **E** were determined by GC-C-IRMS.[1]

The median daidzein and genistein concentration in the urine of the control subjects was 205 and 213 ng/mL, respectively. In contrast the corresponding concentrations for the farm subjects were 1224 and 1966 ng/mL, respectively, thus indicating compliance with the soy diet.

The median $\delta^{13}C$ values for **A** acetates in the control group and farm group were –22.3 and –23.6 $^{o}/_{oo}$, respectively, and that this difference was significant by t-test ($p<0.001$). Similarly the median $\delta^{13}C$ values for **E** acetates in the control group and farm group were –23.7 and –24.9 $^{o}/_{oo}$, respectively, and this difference was significant by t-test ($p<0.001$).

USADA 0903

Figure-2 Typical alteration of delta-values due to **T** doping

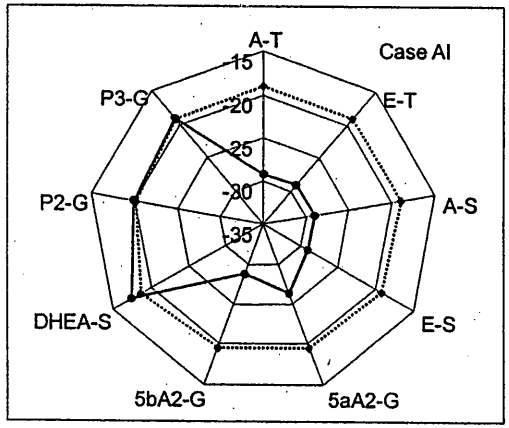


Figure-3 Delta parameters of steroids after DHEA application

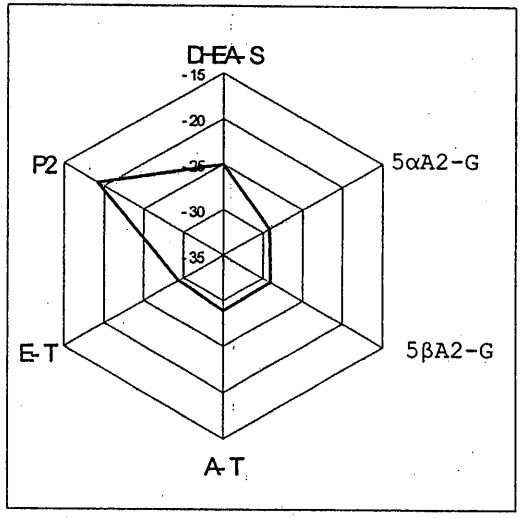
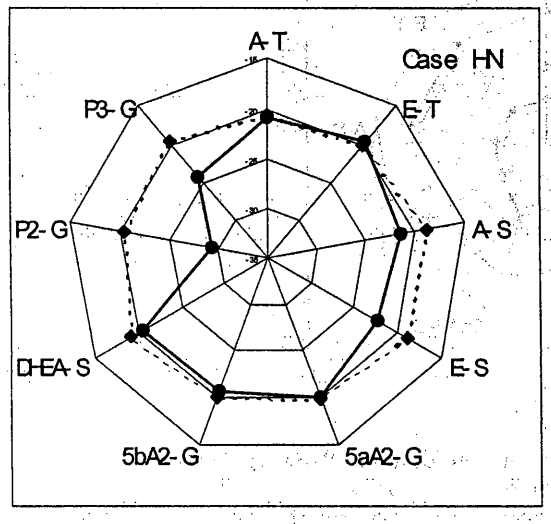


Figure-4 Alteration of delta-values of P2 and P3 after administration of pregnenolone



Question & Answer Discussion

WU
Do you use the oral administration?

UEKI
Yes, it is oral.

WU
Could you inform me of the trademark of the preparation? I know that in our laboratory, we only have the injectable preparation of the T propionate.

UEKI
T propionate is not available in our country. We administer a purified reagent for biochemistry. Why I chose propionate is because it is only effective orally.

SEGURA
Do you have an explanation, when you look at the natural values for your big range -14 to -22?

USADA 0904

UEKI
Probably this high value was caused by the uncertainty of the measurement. What I wanted to show was the difference between these two compounds.

Effect of High Soy Diet on GC-C-IRMS Results

Brian Ahrens
University of California, Los Angeles

Plants may be divided into two main types and several minor types. A C_3 plant takes up CO_2 and incorporates the C atoms into 3-phosphoglycerate or 3-Phosphoglyceric Acid (3-PGA). This is the so-called Calvin cycle of C_3 plants, of which sugar beet is an example. Glucose derived from C_3 plants has a $\delta^{13}C$ of about –25 $^{o}/_{oo}$ or lower. In contrast, when C_4 plants fix CO_2 the fixation yields a C_4-dicarboxylic acid (oxaloacetate). This process is also referred to as the Hatch-Slack cycle. The δ values found in C_4 plants range from about –8 to –20 $^{o}/_{oo}$ (heavier than C_3 plants because they contain more ^{13}C).

Starting materials for the synthesis of **T** are derived from the soy plant. Since soy plants are C_3 plants that are depleted in ^{13}C, we hypothesized that individuals who ingest soy diets will excrete urinary steroids with low ^{13}C values. To test the hypothesis we conducted a cross-sectional study consisting of 18 control subjects and 23 subjects living on a collective farm whose members were purported to subsist solely on soy products. The farm was located in Summertown, TN and the clinical aspects of the study were conducted at the farm and in the neighboring city of Knoxville, TN.

Compliance with the high soy diet was monitored by quantitating soy isoflavones, namely daidzein and genistein, in the urine of the subjects with an LC-MS-MS assay. The delta values of the acetates of **A** and **E** were determined by GC-C-IRMS.[1]

The median daidzein and genistein concentration in the urine of the control subjects was 205 and 213 ng/mL, respectively. In contrast the corresponding concentrations for the farm subjects were 1224 and 1966 ng/mL, respectively, thus indicating compliance with the soy diet.

The median $\delta^{13}C$ values for **A** acetates in the control group and farm group were –22.3 and –23.6 $^{o}/_{oo}$, respectively, and that this difference was significant by t-test ($p<0.001$). Similarly the median $\delta^{13}C$ values for **E** acetates in the control group and farm group were –23.7 and –24.9 $^{o}/_{oo}$, respectively, and this difference was significant by t-test ($p<0.001$).

Figure-2 Typical alteration of delta-values due to **T** doping

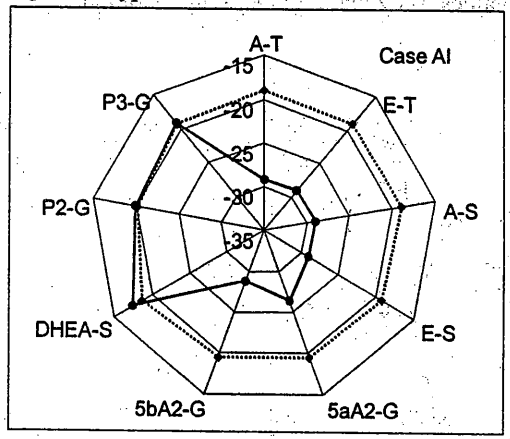
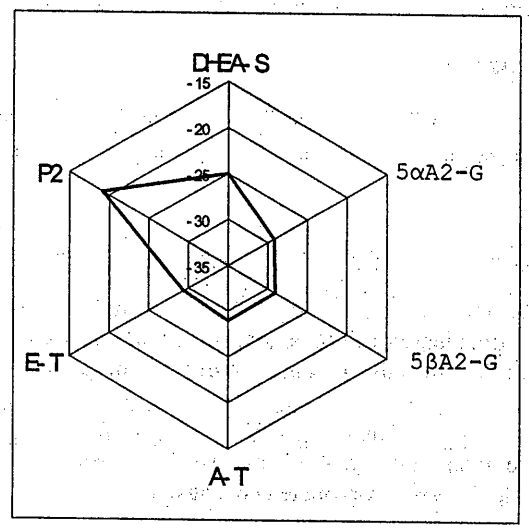


Figure-3 Delta parameters of steroids after DHEA application



M. Donike

Steroid Profiling in Cologne

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

1. Introduction

When it became known to the athletic community that more and more synthetic anabolic steroids could be detected by the comprehensive and sensitive gas-chromatographic / mass-spectrometric screening procedure presented in previous Cologne Workshops and published in 1984 [1], their reaction was to apply endogenous substances, e.g. testosterone and/or human chorionic gonadotropin (HCG). Testosterone was the first endogenous substance used in place of anabolic androgenic steroids. By applying short acting testosterone preparations the time period between final application of anabolic steroids and the time of competition where doping control was expected could also be covered.

The search for a method to detect testosterone misuse was the beginning of steroid profiling in doping analysis. The starting point was the observation [2] that after oral, rectal or intramuscular testosterone application the rate of excretion of testosterone glucuronide increased more than other testosterone metabolites. In excretion studies with testosterone and deuterated testosterone, no change in the excretion rate of epitestosterone was observed. Thus epitestosterone was shown not to be a metabolite of testosterone, and the ratio of testosterone to epitestosterone could thus be used as an indicator for testosterone application.

In 1982, the Medical Commission of the International Olympic Committee (IOC) banned the use of testosterone and defined a ratio of testosterone- to epitestosterone-glucuronide above 6 as an offence of the doping regulations. Since then steroid profiling has been part of the screening procedure for anabolic steroids in Cologne. Androsterone and etiocholanolone are also included in this profile procedure as well as other endogenous steroids [3], so that the quality of the analytical procedure, especially the hydrolysis step, can be monitored.

USADA 0905

A decrease in the amount of endogenous steroids excreted following the application of anabolic androgenic steroids was first observed in samples [4] originating from weightlifters and track and field athletes. This phenomenon was also observed, when in 1985 the International Federation of Bodybuilding (IFBB) banned the use of anabolic steroids and introduced dope controls at major competitions. The extreme misuse of anabolic steroids in this sport revealed the influence of long-term application of anabolic steroids on the endocrine system [5]. It was concluded that monitoring the steroid profile of the athlete may be a valuable method to detect long-term misuse of anabolic steroids. Several approaches to profile monitoring have been explored and results from some of these projects are summarized in this paper.

2. Modifications of the analytical procedure

a) Samples are prepared as described in the protocol published previously [1,3]; however, the aliquot of urine was reduced from 5 ml to 2 ml and the volume of sample injected for analysis was increased from 1 to 3 µl. The advantage is a reduction of the sample preparation time.

b) Further, the urine sample volume prepared is oriented based on its specific gravity. A multiple aliquot of urine is prepared depending on the specific gravity, as shown in Table 1, so that the sensitivity in detecting anabolic steroids is increased. In addition, the quantitation of endogenous steroids, and especially testosterone and epitestosterone, in diluted urine samples is more accurate.

Table 1: Volume of urine prepared based on its specific gravity

urine specific gravity	urine volume of aliquot
> 1.010	2 ml
< 1.010	4 ml
< 1.005	8 ml
< 1.0025	16 ml
< 1.001	20 ml

c) Because of the complex chemistry of the oxidation of dithioerythritol which results in additional peaks in the GC chromatogram, ethanthiol is now used to stabilize the derivatization mixture.

USADA 0906

Table 2: Screening procedure IV protocol for analysis of combined fractions of conjugated and free steroids.

Sample preparation

2 ml of urine and a mixture of internal standards (see Table 2) are added to an Amberlite XAD-2 column. The column (pasteur pipette, closed with a glass pearl, bed height 2 cm) is wahsed with 2 ml of bidistilled water and eluted with 2 ml of methanol.

The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 ml of 0.2 M sodium phosphate buffer pH 7.

50 µl of ß-glucuronidase from E. coli (K12, Boehringer Mannheim) are added to the buffer solution and the mixture is hydrolysed for 1 h at 50°C. The solution is alkalized with 250 µl of 7% potassium carbonate solution to pH 9-10 and the steroids are extracted with 5 ml of diethylether on a mechanical shaker for 5 minutes. After centrifugation the etheral layer is transferred to a glass tube and evaporated to dryness under vacuo.

Derivatization

The dry residue is derivatized with 100 µl of MSTFA/NH_4I/ethanthiol 1000:2:3 (v:w:w) and heated for 15 minutes at 60°C.

GC/MS parameters

GC/MS HP 5996
carrier gas: helium, 1 ml/min at 180°C, split 1:10
column: HP Ultra-1 fused silica, crosslinked methyl silicone (OV 1), 17 m, 0.2 mm i.D., 0.11 µm film thickness
temperature program: 180°C; 3°C/min - 231°C; 30°C/min - 310°C

2.3 Quantitation

Methyltestosterone was first introduced as an internal standard when at that time the main interest was to quantify the amount of testosterone and epitestosterone excreted. In extending the steroids of interest to include androsterone, etiocholanolone, 11ß-hydroxyandrosterone and 11ß-hydroxyetiocholanolone it was found that the stability of the calibration factors with methyltestosterone as internal standard was not satisfying. Figure 1 shows the shift in the calibration factors for the target steroids in 35 calibration mixtures measured under routine conditions from January through May 1992 calculated with methyltestosterone as an internal standard. It is obvious that most changes in the

calibration factors are parallel and can be attributed to the variation of the peak area of methyltestosterone.

A logical approach to reduce the variation in calibration factors for the target steroids was to introduce deuterated internal standards at concentration in the range of the respective steroids. The deuterated standards in Table 2 were synthesized [6] and introduced into the profiling procedure.

Table 2: Internal standards used in the quantification of endogenous steroids.

internal standard	concentration [ng/ml]	target compound
methyltestosterone	500	all registered endogenous steroids
[2,2,4,4-$^2H_4$]-etiocholanolone	500	androsterone, etiocholanolone
[16,16,17-$^2H_3$]-testosterone	40	testosterone, epitestosterone
[2,2,4,4-$^2H_4$]-11ß-hydroxyandrosterone	240	11ß-hydroxy-androsterone, 11ß-hydroxy-etiocholanolone

Figure 2 shows the calibration factors calculated with the respective deuterated substance as internal standards. As can be seen from Table 3, the coefficient of variation is minimized when using the respective deuterated steroid as an internal standard for calculation of the calibration factors.

USADA 0907

Table 3: Calibration factors for the quantification of characteristic endogenous androgenic steroids calculated with different internal standards: -M = methyltestosterone -E = d4-etiocholanolone -T = d3-testosterone -O = d4-11ß-hydroxy-androsterone

	N	Min	Max	Mean	St. Dev.	CV %
ANDR-M	35	.68	1.11	.838	.112	13.37
ETIO-M	35	.72	1.18	.893	.120	13.44
EPIT-M	35	1.75	3.00	2.439	.353	14.47
TEST-M	35	1.79	3.16	2.537	.359	14.15
OHA-M	35	.42	.78	.565	.101	17.88
OHE-M	35	.33	.63	.460	.081	17.61
ANDR-E	35	1.03	1.38	1.213	.097	**8.00**
ETIO-E	35	1.13	1.46	1.293	.100	**7.73**
EPIT-E	35	2.71	4.67	3.540	.462	13.05
TEST-E	35	2.76	4.85	3.684	.486	13.19
OHA-E	35	.66	1.12	.820	.143	17.44
OHE-E	35	.51	.91	.669	.119	17.79
ANDR-T	35	.35	.56	.440	.062	14.09
ETIO-T	35	.37	.61	.470	.067	14.26
EPIT-T	35	1.02	1.49	1.276	.143	**11.21**
TEST-T	35	1.08	1.54	1.325	.131	**9.89**
OHA-T	35	.24	.44	.295	.052	17.63
OHE-T	35	.20	.34	.241	.038	15.77
ANDR-O	35	1.67	3.32	2.387	.482	20.19
ETIO-O	35	1.84	3.55	2.549	.533	20.91
EPIT-O	35	4.85	9.49	6.938	1.399	20.16
TEST-O	35	5.09	9.66	7.203	1.354	18.80
OHA-O	35	1.25	1.74	1.575	.157	**9.97**
OHE-O	35	1.03	1.44	1.283	.123	**9.57**

3. Testosterone / epitestosterone ratio

A prominent change in the steroid profile can be observed after testosterone application. The reason is that the amount of testosterone-17-glucuronide excreted after testosterone application increases by at least an order of magnitude more than any of the testosterone metabolites (Figure 3 and Table 4).

To examine the metabolic pathways, an excretion study with 50 mg 1,2-dideutero-testosterone [7] was performed. In Table 4 the results of this excretion study are compared with excretion rates of some testosterone metabolites from a reference population [8,9].

The excretion rate for 1,2-dideutero-testosterone increased in the first 7 hours by a factor of 147 whereas epitestosterone only increased by a factor of 2. This result with labelled testosterone confirmed the observation that the epitestosterone concentration was not influenced by testosterone application. In addition it established the selection of epitestosterone as an "endogenous internal standard" to detect application of testosterone.

Smaller compared to the increase in excreted testosterone were the relative increases of androsterone and etiocholanolone, about 8 and 10 respectively. A consequence of this increase is that the ratio of androsterone to testosterone (A/T) decreases and this can be used as additional parameter to detect application of testosterone (see 4.1). In the above described experiment the A/T ratio following application of testosterone was only 3.4 while in the reference population the A/T ratio was 63..

Table 4: Metabolism of testosterone, 50 mg 1,2-dideutero-testosterone, applied orally.

Steroid	median of the reference population* µg/h (0-7 h p.a.)	dideutero-metabolites µg/h (0-7 h p.a.)	factor exogenous/endogenous
Androsterone	144	1140	7.9
Etiocholanolone	106	1106	10.4
Epitestosterone	2.1	4.1	2.1
Testosterone	2.3	339	147.4

* 105 sport students [8,9]

4. Steroid profile in doping analysis

Until now, doping analysis has been limited to the detection of banned substances or their metabolites. The use of the steroid profile as a tool to detect the misuse of anabolic steroids has been difficult since one must first define standard or "normal" values. Nevertheless, steroid profiling can serve to examine the endogenous status of an athlete. The following examples will be discussed:

1. development of the steroid profile in weightlifting.
2. steroid profile atlas for the German decathlon team.
3. longitudinal monitoring of elevated T/E ratios.

4.1 Weightlifting World Championships 1989 and 1990

After the disaster of the 1988 Olympic Games in Seoul with 5 positive athletes, two of which were suspended gold medalists, the International Weightlifting Federation (IWF) took strict measures to fight against the misuse of anabolic steroids. The number of samples taken in and out of competition was increased and all samples were analysed in Cologne in order to compare results with an existing data base and to establish a data base with the steroid profiles of weightlifters.

After evaluation of the results of 1989, the IWF Executive Board and the IWF Congress decided to take appropriate sanctions when the steroid profile showed deviations from the "norm" in order to extend the deterrent effects of doping controls at and out of competition. This regulation became effective for the 1990 World Championships in Budapest. Table 5 shows the text defining a non-acceptable steroid profile. The reference limits were calculated using routine doping control samples from cyclists measured in 1988 and 1989 in Cologne [9].

USADA 0908

Table 5: Excerpt from regulations of the IWF regarding the steroid profile.

> The quantitative determination will be performed on 3 aliquots of the urine sample, each aliquot injected at least twice into the gas-chromatograph / mass spectrometer.
>
> Criteria for a non acceptable steroid profile will be that the first three values of the following parameters are lower than the reference limits given in the table below, or the first, the third and the fourth lower than specified:

Parameter	limit
1. Androsterone [ng/ml]	766,4
2. Etiocholanolone [ng/ml]	700,9
3. Epitestosterone [ng/ml]	8,2
4. Androsterone/Etiocholanolone	0,45

Table 6 shows the application of these reference limits on the samples obtained during the Weightlifting World Championships in 1989 and 1990. In 1989, 15 athletes fulfilled the criteria and would have been banned, whereas in 1990, none of the athletes fulfilled the criteria. Of special (biochemical) interest is that not only the concentrations of the endogenous steroids normalized, but also the androsterone / etiocholanolone ratio (A/E), which means that the activity of the 5α-reductase returned to normal.

Table 6: Application of the reference limits on the samples of the Weightlifting World Championships (WCH) 1989 (n=84) and 1990 (n=96).

	WCH 1989 n	WCH 1990 n
Androsterone < 766.4	19	0
Etiocholanolone < 700.9	22	4
Epitestosterone < 8.2	23	5
Androsterone/Etiocholanolone < 0.45	1	0
three criteria fulfilled	15	0

The improvement of the steroid profiles of the weightlifter population can be statistically demonstrated as a significant increase in the concentrations as shown in Figure 4.

4.2 German decathlon team

The German decathlon team decided to underline their attitude against doping by agreeing to give a urine sample every two weeks as an out-of-competition doping control with the goal of establishing a steroid profile atlas for each athlete. Figures 5 and 6 show the variability of the androsterone/etiocholanolone (A/E) and the testosterone/epitestosterone (T/E) ratios as results of a preliminary evaluation. None of the steroid profiles was suppressed or showed changes in the ratios T/E or A/E over the time period investigated.

4.3. Elevated T/E-ratios: longitudinal monitoring and suppression studies

During the past years, it occurred on several occasions that an athlete with a testosterone/epitestosterone ratio above 6 claimed not to have misused testosterone, its precursors, HCG, or anabolic steroids. The high T/E ratio in these cases is often caused by a low epitestosterone concentration which may be endogenous or induced. One possible approach to follow up such cases is a longitudinal monitoring of the steroid profile of this athlete, best under quarantaine conditions, second best by out-of-competition controls on short notice.

A second approach was described by Oftebro [10]. Ketoconazol, a cytochrome P_{450} inhibitor, is administered to suppress the testicular testosterone production. Based on our experience, a single dose of ketoconazol, 6 mg/kg bodyweight, allows one to differentiate between a high T/E ratio due to the application of testosterone, or a naturally elevated T/E ratio caused by a relative low epitestosterone concentration.

A case study with a cyclist who on several occasions showed a T/E ratio of about 6 or higher is presented. Figure 7 shows the T/E ratio, testosterone and epitestosterone concentration of this athlete throughout the year 1991. The T/E ratio lies within the range of 5.1 to 8.4 (mean = 6.9, stdv = 1.2) and a coefficient of variation of 17%. This coefficient of variation lies within the order of magnitude of individual variations, a first hint to a "normally" elevated T/E ratio. A further cause is the low epitestosterone concentration.

USADA 0909

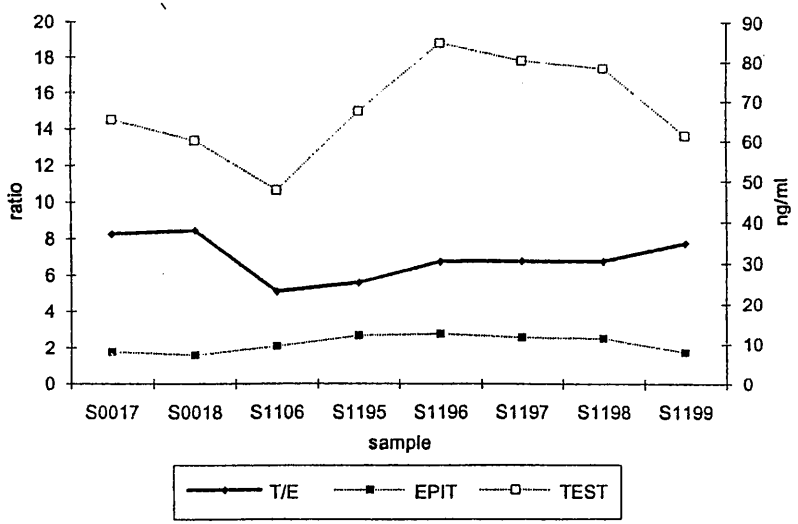


Figure 7: T/E ratio, and urinary testosterone and epitestosterone concentrations in several samples from a cyclist throughout the year 1991.

In order to evaluate the steroid profile, three experiments were performed:

1. urine collection over 60 hours, 10 spot samples plus 2 pooled samples overnight
2. a depression study of the testis with ketoconazol
3. a depression study of the adrenal gland with dexamethasone.

Figures 8 shows the results of the samples collected over the 60 hour period. The epitestosterone concentration is fairly constant, but low, wherease the testosterone concentration is much higher and fluctuates to a larger extent. The T/E ratio varies between 6 and 14 (mean=8.89, stdv=1.97). The coefficient of variation of the T/E ratio is 22% and lies well within the range found in a study on the longterm stability of the steroid profile [11] and the coefficient of variation observed on this subject previously (compare Figure 7).

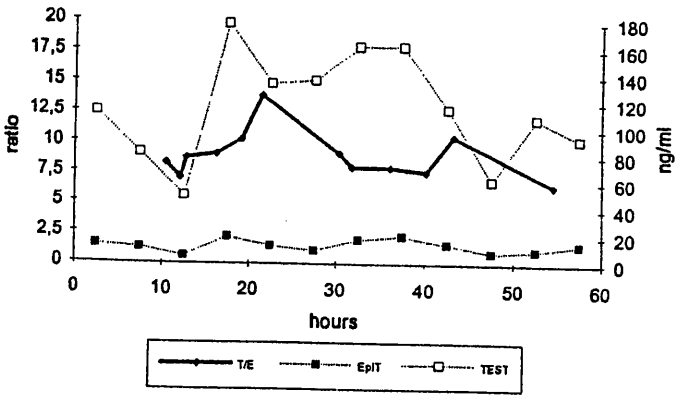


Figure 8: T/E ratio, and testosterone and epitestosterone concentations of 12 samples from a cyclist provided consecutively over 3 days.

USADA 0910

In the testicular testosterone suppression study, four hours after application of ketoconazol, both the testosterone and epitestosterone concentrations were significantly lower than the starting values. The testosterone excretion was suppressed to a higher extent than the epitestosterone excretion resulting in a decrease of the T/E ratio from about 6 to about 2 (Figure 9). The depression of the testosterone excretion would not be observed in the case of exogenously applied testosterone.

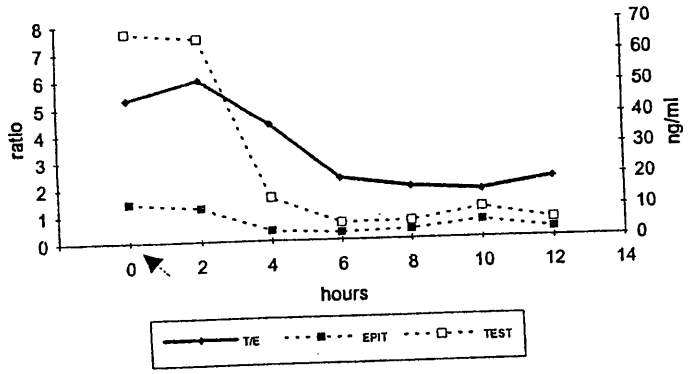


Figure 9: T/E ratio, and testosterone and epitestosterone concentrations in samples delivered from a cyclist after application of ketoconazol, 2 x 200 mg orally at t=0.

Depression of the adrenal gland by application of a synthetic corticosteroid, dexamethasone, did not influence the rate of testosterone excretion, but it did seem to depress slightly the epitestosterone excretion rate. The T/E ratio increased to a small extent, but the fluctuation paralleled the testosterone concentration.

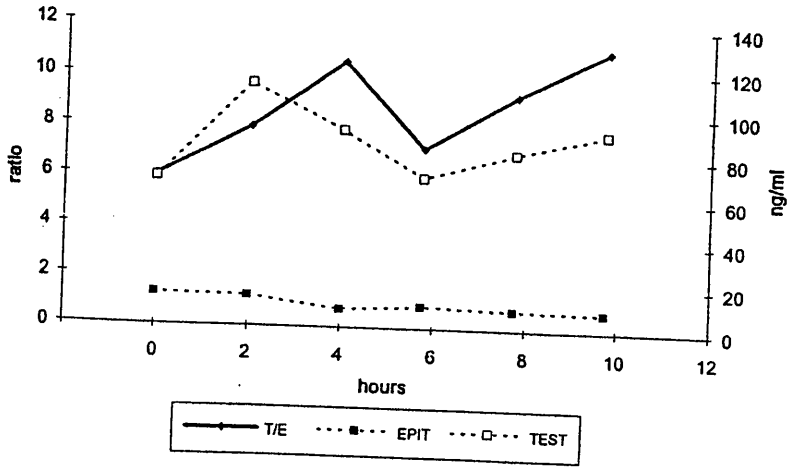


Figure 10: T/E ratio, and urinary testosterone and epitestosterone concentrations in samples from a cyclist delivered after the application of dexamethasone, 3 mg orally at t=0.

In discussion of the origin of epitestosterone, the ketoconazol and dexamethasone experiments indicate that the testis secretes epitestosterone or its precursors to a larger extent than the adrenal gland.

USADA 0911

Summary

Steroid profiling is a valuable tool when performed routinely on urinary doping control samples under carefully controlled analytical conditions. The details of the analytical procedure especially the quantitation using four different internal standards, three of them deuterated, are presented.

The steroid profiles of an individual without interference from anabolic androgenic steroids or inhibitors of enzymes are relative stable. The most prominent change in the steroid profile is observed after testosterone application, which will lead to a high dose dependent increase of the testosterone-glucuronide. The suppression of the endogenous production of androgenic steroids by anabolic steroids is well known. Therefore the application of lower reference limits by the International Weightlifting Federation is presented as an example. Further examples for a meaningful application of steroid profiling are longitudinal studies on German decathlon athletes, and the differentiation between high T/E ratios caused by endogenous low epitestosterone concentrations or by testosterone administration.

USADA 0912

References

1. M.Donike, J.Zimmermann, K.-R.Bärwald, W.Schänzer, V.Christ, K.Klostermann and G.Opfermann
 Routinebestimmung von Anabolika in Harn
 Deutsche Zeitschrift für Sportmedizin, 35 (1984) 14-24.

2. M. Donike, K.-R. Bärwald, K. Klostermann, W. Schänzer, and J. Zimmermann
 Nachweis von exogenem Testosteron
 in: Sport: Leistung und Gesundheit. Edt.: H.Heck, W.Hollmann, H.Liesen, R.Rost, Deutscher Ärzte Verlag Köln, (1983) 293.

3. M. Donike, H. Geyer, A. Gotzmann, M. Kraft, F. Mandel, E. Nolteernsting, G. Opfermann, G. Sigmund, W. Schänzer, and J. Zimmermann
 Dope analysis
 in: P.Belotti, G.Benzi, A.Ljungqvist (eds.)
 Official proceedings. Ist International Athletic Foundation World Symposium on Doping in Sport, Florenz 1987
 International Athletic Foundation, Florence (1988) 53-80.

4. M.Donike, H.Geyer, W.Schänzer, J.Zimmermann
 Die Suppression der endogenen Androgenproduktion durch Metandienon
 in: Training und Sport zur Prävention und Rehabilitation in der technisierten Umwelt. Eds.: I.-W.Franz, H.Mellerowicz, W.Noack, Springer Verlag Berlin - Heidelberg - New York -
 Tokyo, (1985) 508.

5. M. Donike, H. Geyer, M. Kraft, and S. Rauth
 Longterm Influence of Anabolic Steroid Misuse on the Steroid Profile.
 in: P.Belotti, G.Benzi, A.Ljungqvist (eds.)
 Official proceedings. IInd International Athletic Foundation World Symposium on Doping in Sport, Monte Carlo 1989
 International Athletic Foundation, Monte Carlo (1990) 107-116.

6. W. Schänzer et al.
 to be published

7. J. Zimmermann
Untersuchungen zum Nachweis von exogenen Gaben von Testosteron
Thesis Deutsche Sporthochschule Köln, Hartung-Gorre Verlag Konstanz, 1986.

8. A. Wolansky
Androgene Steroide - Die Referenzbereiche der Konzentrationen und Ausscheidungsraten
Diplom Thesis Deutsche Sporthochschule Köln, 1992.

9. M. Donike, S. Rauth und A.Wolansky
Reference Ranges of Urinary Endogenous Steroids determined by Gas-Chromatography / Mass Spectrometry
in: Proceedings of the 10th Cologne Workshop on Dope Analysis, 7th till 12th June 1992, in press.

10. H. Oftebro
Evaluating an abnormal urinary steroid profile
The Lancet, 339 (1992) 941.

11. U. Mareck-Engelke, H. Geyer and M. Donike
Stability of Steroid Profiles
in: Proceedings of the 10th Cologne Workshop on Dope Analysis, 7th till 12th June 1992, in press.

USADA 0913

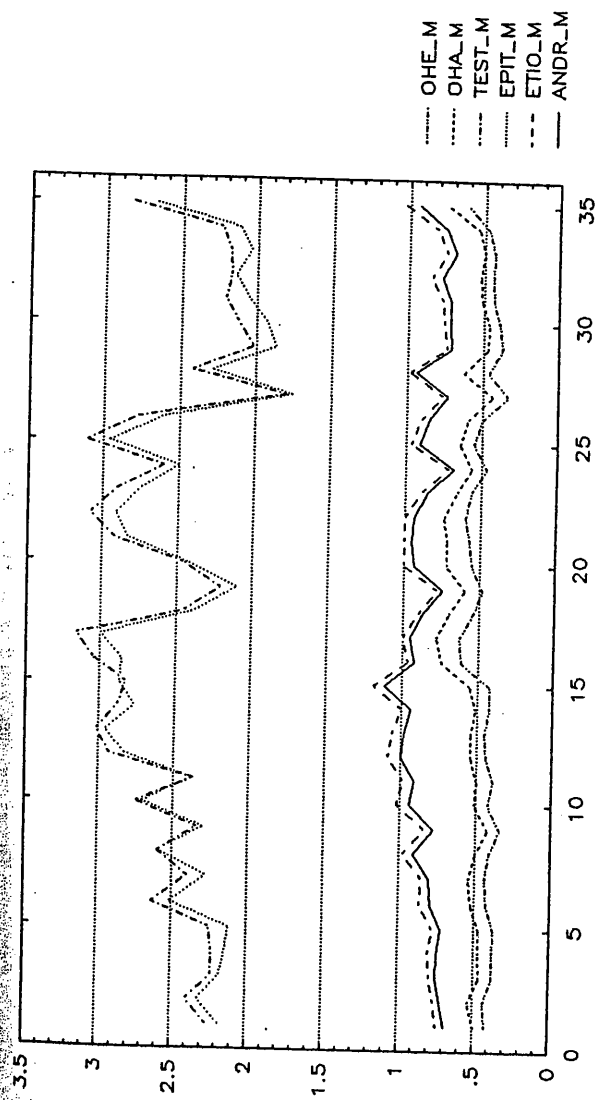


Figure 1: Shift in the calibration factors calculated with methyltestosterone as internal standard in pairs epitestosterone and testosterone (EPIT_M, TEST_M), androsterone and etiocholanolone (AND_M, ETIO_M), 11ß-hydroxy-androsterone and 11ß-hydroxy-etiocholanolone (OHA_M, OHE_M)

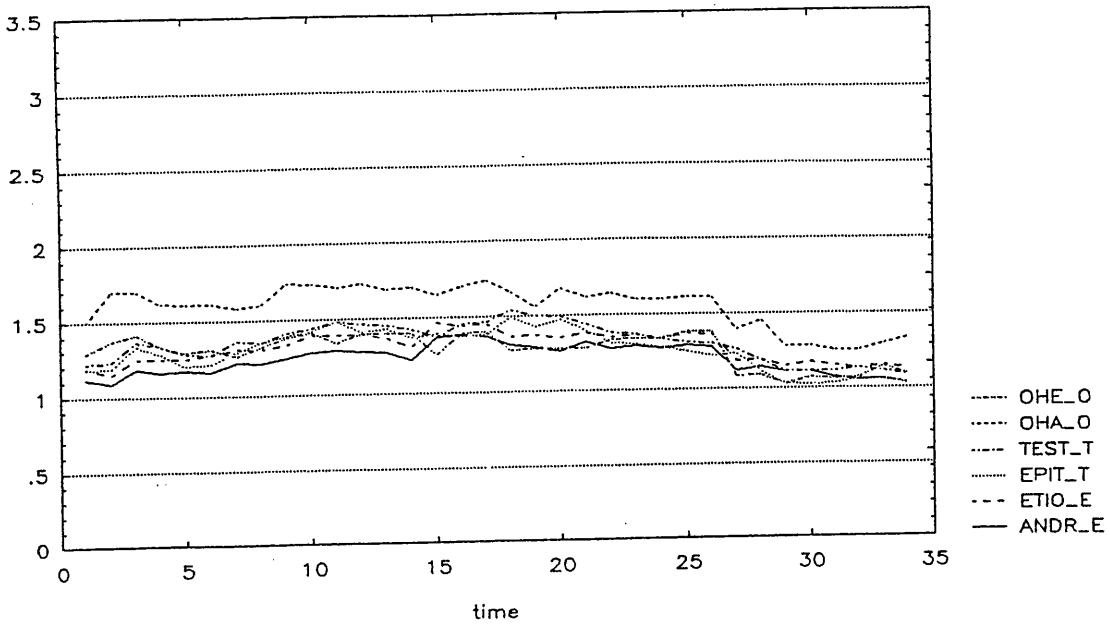


Figure 2: Calibration factors calculated with three deuterated internal standards. Androsterone and etiocholanolone with d4-etiocholanolone; epitestosterone and testosterone with d3-testosterone; and 11ß-hydroxy-androsterone and 11ß-hydroxy-etiocholanolone with d4-11ß-hydroxy-androsterone.

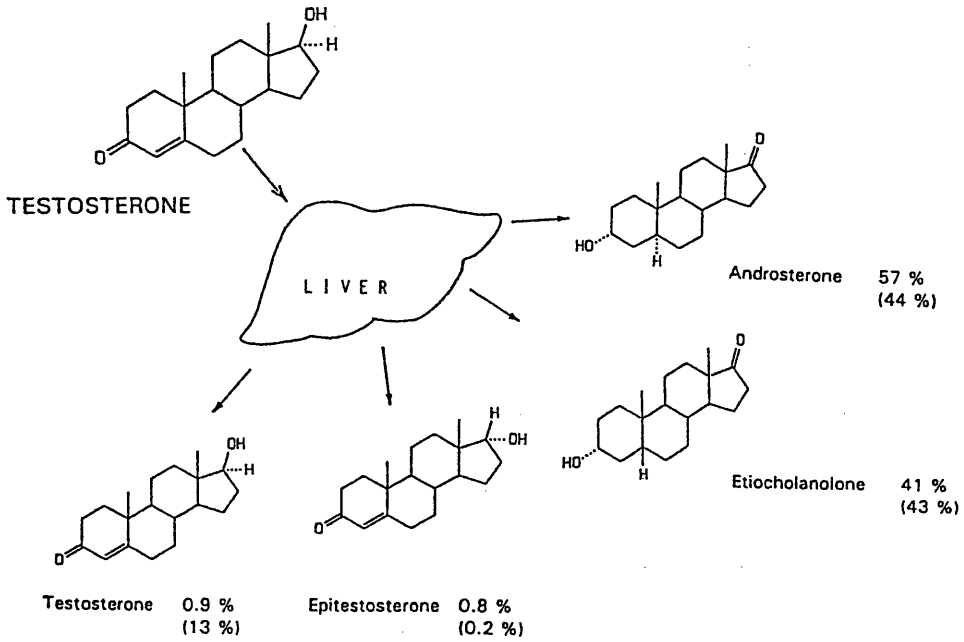


Figure 3: Relative excretion of testosterone metabolites, normally and after application of dideutero-testosterone (in brackets).

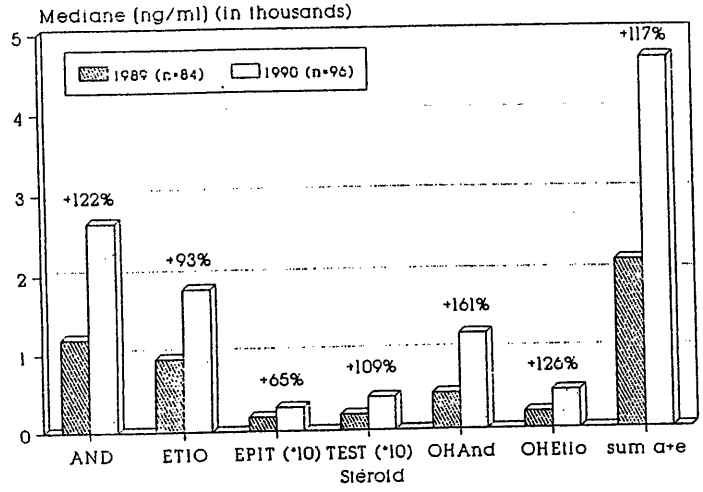


Figure 4: Mediane of the steroid concentrations of androsterone (AND), etiocholanolone (ETIO), epitestosterone (EPIT) times 10, testosterone (TEST) times 10, 11ß-hydroxy-androsterone (OHAnd), 11ß-hydroxy-etiocholanolone (OHEtio), and the sum of androsterone and etiocholanolone (sum a+e) of the participants of the Weightlifting Worldchampionships 1989 and 1990. All differences are highly significant.

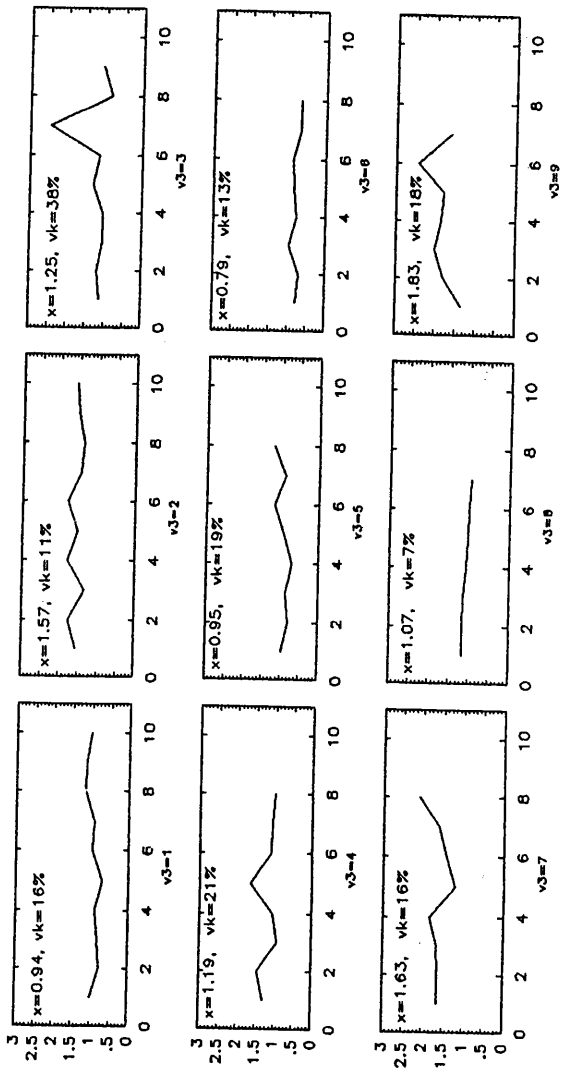


Figure 5: Androsterone/etiocholanolone ratio of 9 athletes of the German Decathlon Team, up to 10 controls within 6 months. x = mean, vk = coefficient of variation.

USADA 0915

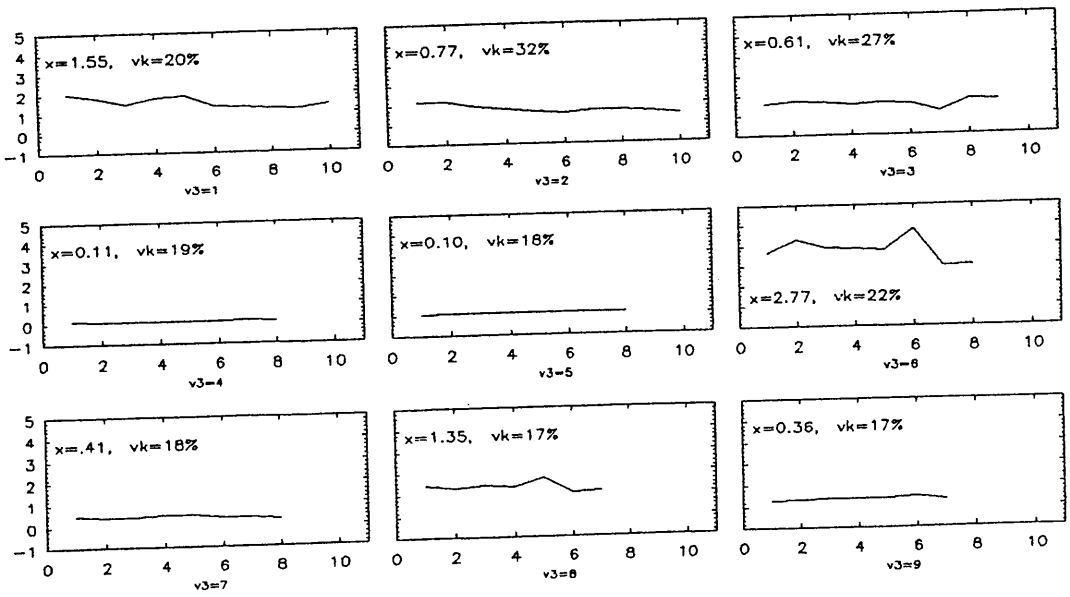


Figure 6: Testosterone/epitestosterone ratio of 9 athletes of the German Decathlon Team, up to 10 controls within 6 months. x = mean, vk = coefficient of variation.

USADA 0916

M. Donike, S. Rauth und A.Wolansky

Reference Ranges of Urinary Endogenous Steroids Determined by Gas-Chromatography / Mass Spectrometry

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

1. Introduction

The administration of testosterone or its chemical derivatives, the anabolic androgenic steroids, may change dramatically the excretion of the urinary endogenous steroids most of them are endproducts of the steroid metabolism. In the case of application of synthetic testosterone independent of the route of administration the most prominent change in the urinary steroid profile is the increase of testosterone glucuronide [1, 2]. This observation inititated as early as 1982 [3] a study to determine the normal range for testosterone and epitestosterone concentrations as well as the testosterone/epitestosterone ratio [4].

Following the administration of the anabolic steroid metandienone to males, the androgen excretion was reduced substantially, an observation which is in accordance with the well-known feedback mechanism regulating the testosterone production by the hypothalamic-pituitary-gonadal axis [5]. With the increase of the number of dope controls in some sports in which the misuse of anabolic steroids is well-known, steroid profiles were found which were characterized by low concentrations of the endogenous urinary metabolites and often by a low androsterone/etiocholanolone ratio [6]. It is obvious that the suppression and alteration of the biosynthesis of the endogenous steroid hormones lasts longer than the analytical detectibility of the administered synthetic anabolic steroids and their metabolites. Therefore efforts were undertaken to determine reference values for the concentrations e.g. of androsterone, etiocholanolone, and ratios e.g. androsterone / etiocholanolone, androsterone / testosterone, characteristic for the steroid profile.

USADA 0917

2. Materials and methods

2.1 Analysis

Determination of the endogenous steorids was performed as described earlier [7] with the following modifications:

a) 2 ml of urine was prepared for hydrolysis
b) 200 ng methyltestosterone was added as internal standard (population of cyclists) 1000 ng methyltestosterone was added as internal standard (population of sport students)
c) helium was used as GC carrier gas.
d) the calculated urine concentrations of the endogenous steroids were corrected using a specific gravity of 1.020 as described earlier [8]

2.2 Selection of reference populations

2.2.1 Amateur cyclists

The amateur cyclists samples were doping control samples collected by the German Cycling Federation (BDR) during amateur cycling road races in the years 1988 and 1989. In 1988 the samples included 235 male athletes in 1989 247 male athletes. The analytical results of the samples were included in the data base when the following criteria were met:

a) reproducible gas-chromatographic/mass spectrometric conditions - internal standard identified and giving a peak area in the expected intensity
b) identification of internal standard with integrated peak area within an accepted range
c) correct integration of the endogenous steroids ion chromatogram signals.

2.2.2 Sport students

120 male sport students volunteered to take part in a study to determine some parameters of the steroid profile. After voiding the bladder at 1 p.m., they were asked to provide a urine specimen after exactly 3 hours. 15 urine samples were excluded from the statistical evaluation because one of the following criteria was met: The student was non caucasian, the student had previously or currently used anabolic steriods or drugs, and the delivered urine volume was diluted or concentrated (calculated by an urine flow lower than 0.4 or higher than 1.7 ml/min).

USADA 0918

Volunteers included in this study were healthy, had no medical history of cardiovascular, renal, gastrointestinal, liver, endocrine, or testicular desease respectively injuries.

Table 1: Parameters of the reference population (n=105, male)

	age [years]	height [cm]	weight [kg]
mean	24.4	182.7	76.8
min	19	170	62.0
max	30	199	100
stdev	1.96	5.69	7.77

2.3 Statistical evaluation

Mean, standard deviation, median and range as well as the t-test for independent samples were calculated with CSS Statistica [9]. The 95% reference ranges were calculated with REFVAL [10] using the non parametric percentile method which includes confidence intervals for the reference vaulues. This statistical approach was recommended by the Expert Panel on Theory of Reference Values of the International Federation of Clinical Chemistry (IFCC) and the Standing Committee on Reference Values of the International Committee for Standardization in Haematology (ICSH) [11].

3. Results

The analytical results obtained from the two populations of amateur cyclists and sport students and the calculated reference ranges (using the IFCC recommended statistical approach) are summarized in the following Tables 2 through 7.

The controlled study with the sport students, enables the calculation of excretion rates (µg/h) which are presented in Table 6.

3.1 Amateur cyclists

Table 2.1: Endogenous steroid concentrations, amateur cyclists (n=482), descriptive statistics.

steroid concentration	mean ng/ml	minimum ng/ml	maximum ng/ml	median ng/ml	stdv
androsterone (A)	2474	459	9385	2164	1425
etiocholanolone (E)	2272	354	9702	1905	1349
epitestosterone (EPI)	37	3.4	203	30	26
testosterone (T)	42	1.5	220	38	28
11ß-hydroxy-androsterone (OHA)	858	29	6955	623	787
11ß-hydroxy-etiocholanolone (OHE)	412	17	2991	282	410
sum A+E+Epi+T+OHA+OHE	6092	1433	25774	5222	3320

Table 2.2: Endogenous steroid concentration ratios, amateur cyclists (n=482), descriptive statistics.

steroid ratio	mean	minimum	maximum	median	stdv
T/E	1.43	0.05	7.15	1.23	1.00
A/E	1.2	0.32	4.61	1.12	0.52
OHA/OHE	3.24	0.23	17.5	2.12	3.17
A/T	99	9.97	1350	59	145
A/Epi	86	6.7	371	71	57
E/T	93	9.98	1424	54	155
E/Epi	80	9.02	413	67	56

Table 3.1: Reference ranges of endogenous steroid concentrations, amateur cyclists (n=482).

steroid	95% reference limits ng/ml		90% confidence intervals ng/ml	
	lower limit	upper limit	lower interval	upper interval
androsterone (A)	766.4	6673.1	690.3 - 857.2	5475.8 - 6758.7
etiocholanolone (E)	700.9	5738.7	623.8 - 759.1	5050.1 - 6185.3
epitestosterone (Epi)	8.2	113.2	6.8 - 9.9	95.9 - 121.5
testosterone (T)	3.8	121.7	3.2 - 4.5	100.8 - 135.2
11ß-hydroxy-androsterone (OHA)	95	2804	79 - 119	2410 - 3627
11ß-hydroxy-etiocholanolone (OHE)	31	1655	22 - 44	1326 - 1879
sum A+E+Epi+T+OHA+OHE	2019	15021	1897 - 2232	13073 - 16593

Table 3.2: Reference ranges of endogenous steroid concentration ratios, amateur cyclists (n=482).

steroid ratio	95% reference limits ng/ml		90% confidence intervals ng/ml	
	lower limit	upper limit	lower interval	upper interval
T/Epi	0.1	4.0	0.08 - 0.13	3.5 - 4.4
A/E	0.45	2.38	0.41 - 0.51	2.23 - 2.67
OHA/OHE	0.46	13.11	0.33 - 0.53	12.21 - 15.14
A/T	17.95	556.1	14.86 - 20.59	402.9 - 632.8
A/Epi	19.15	239.9	17.46 - 21.91	204.3 - 268.3
E/T	16.33	481.9	13.42 - 17.47	329.5 - 922.7
E/Epi	15.61	238.1	13.05 - 18.24	211.1 - 258.3

USADA 0919

3.2 Sport students

Table 4.1: Endogenous steriod concentrations, sport students (n=105), descriptive statistics.

steroid	mean ng/ml	minimum ng/ml	maximum ng/ml	median ng/ml	stdv
androsterone (A)	3051	915	9162	2879	1424
etiocholanolone (E)	2166	487	6211	1989	946
epitestosterone (EPI)	50	6.6	210	41	36
testosterone (T)	52	2.6	153	44	35
11ß-hydroxy-androsterone (OHA)	1098	308	3650	989	583
11ß-hydroxy-etiocholanolone (OHE)	322	21	2051	240	292
sum A+E+Epi+T+OHA+OHE	6738	2121	18891	6303	2772

Table 4.2: Endogenous steriod concentration ratios, sport students (n=105), descriptive statistics.

steroid ratio	mean	minimum	maximum	median	stdv
T/EPI	1.37	0.02	5.89	1.05	1.07
A/ETIO	1.51	0.47	4.11	1.40	0.62
OHA/OHE	6.0	0.71	40	4.23	6.40
A/T	130	18.92	1515	61.63	221
A/EPI	81.1	13.10	330	66.80	51.22
E/T	83.5	11.2	626	47.05	116
E/Epi	60.6	7.69	244	49.72	42.86

Table 5.1: Reference ranges of endogenous steroid concentrations, sport students (n=105).

steroid	95% reference limits ng/ml		85% confidence intervals ng/ml	
	lower limit	upper limit	lower interval	upper interval
androsterone (A)	1157	7551	915 - 1418	5273 - 9162
etiocholanolone (E)	726	4452	487 - 1008	3883 - 6211
epitestosterone (EPI)	9.3	154	6.6 - 14.2	135 - 210
testosterone (T)	3.4	148	2.6 - 4.5	126 - 153
11ß-hydroxy-androsterone (OHA)	346	2936	308 - 486	2178 - 3650
11ß-hydroxy-etiocholanolone (OHE)	42	1001	21 - 55	749 - 1334
sum A+E+Epi+T+OHA+OHE	2792	15751	2121 - 3569	11273 - 19892

Table 5.2: Reference ranges of endogenous steroid concentration ratios, sport students (n=105).

steroid ratio	95% reference limits		85% confidence intervals	
	lower limit	upper limit	lower interval	upper interval
A/E	0.58	3.28	0.47 - 0.74	2.8 - 4.1
T/Epi	0.06	4.40	0.02 - 0.11	3.3 - 5.9
OHA/OHE	0.84	31.30	0.71 - 1.23	14 - 40
A/T	20	738	19 - 27	599 - 920
A/Epi	25	237	13 - 30	185 - 330
E/T	14	492	11 - 17	368 - 653
E/Epi	15	203	7.7 - 18	133 - 244

USADA 0920

Table 6: Endogenous steroid excretion rates, sport students (n=105), descriptive statistics.

steroid excretion rates	mean µg/h	minimum µg/h	maximum µg/h	median µg/h	stdv
androsterone (A)	148	36.3	300	144	51
etiocholanolone (E)	108	29.2	240	106	43
epitestosterone (EPI)	2.4	0.3	8.5	2.1	1.59
testosterone (T)	2.6	0.1	7.5	2.3	1.58
11ß-hydroxy-androsterone (OHA)	53	15.1	125	49.5	21.6
11ß-hydroxy-etiocholanolone (OHE)	16	1.0	74	12	13.2

Table 7: Reference ranges of endogenous steroid excretion rates, sport students (n=105).

steroid	95% reference limits µg/h		85% confidence intervals µg/h	
	lower limit	upper limit	lower interval	upper interval
androsterone (A)	47	267	36 - 74	225 - 300
etiocholanolone (E)	34	227	29 - 43	190 - 240
epitestosterone (EPI)	0.46	7.98	0.30 - 0.70	5.1 - 8.5
testosterone (T)	0.17	6.59	0.09 - 0.20	5.5 - 7.5
11ß-hydroxy-androsterone (OHA)	21	114	5 - 25	93 - 125
11ß-hydroxy-etiocholanolone (OHE)	1.59	53	1.1 - 2.7	40 - 74

USADA 0921

4. Discussion

4.1 General

The term "reference range" was introduced by Gräsbeck and Saris [12] in order to replace the term "normal range" as the definition of normal is difficult and unprecise. Reference values are generated from a defined population, using a defined protocol for sample taking, and a defined analytical method which has a long-term stability monitored by quality control. So results of an individual, when generated under the same sampling and analytical conditions, can be interpreted using reference values of a representative population.

In doping analysis deviations of an exact protocol are encountered as there is a lack of detailed information regarding age, weight, training history, time of day, physical strain and sometimes even the sport discipline. An additional disadvantage is the fact that most of the urine samples collected for doping control purposes are spot samples, and as such no correlation between measured steroid concentrations and urinary excretion rates can be established.

A further point of discussion is the best statistical approach. Three principle methods are proposed [14]:

1. non-parametric approaches which are distribution independent e.g. the determination of tolerance intervals or percentiles;
2. transformation of the data to obtain a Gaussian distribution;
3. fitting a distribution function to the data.

The Expert Group of the IFCC proposes two methods: the non parametric percentile method or a two step transformation to the Gaussian distribution. In both cases confidence intervals for the reference values can be calculated. Studies performed do not confirm an advantage of either method [15]. The data presented in this paper are calculated using the percentile method, as not all data could be transformed to Gaussian distribution.

A minimum of 300 samples is recommended [13] to obtain statistically significant results. As such the reference ranges of the cyclists (n=482) should be prefered. In addition, the small sample size of the sport students (n=105) allows one to calculate only an 85% confidence interval.

4.2 Selecting reference populations

One important factor in calculating reference ranges for endogenous steroids is the influence of drugs, especially anabolic steroids, administered therapeutically or abused. The population of german amatuer cyclists was chosen as a reference population because of the background information available: The German Cycling Federation "Bund Deutscher Radfahrer" (BDR) performs doping controls at all important events throughout the whole cycling season, i.e. up to 12 months a year. We assume that because of this frequent testing the misuse of anabolic steroids within the group of cyclists participating in races in Germany is negligable. This hypothesis is supported by the fact that out of about 900 samples analysed for the BDR 1988 and 1989 only one was positive with an anabolic steroid (clostebol).

As there are contradictory reports in the literature regarding the influence of physical workload on the secretion of testosterone and other androgenic steroids, in addition to the cyclist population, reference values were generated from a population of sport students who did not perform any excercise on the day of sample collection.

4.3 Statistical results

The endogenous steroid concentrations and ratios of the cyclist and sport student population were compared statistically using the t-test for independant samples. The concentrations of all measured steroids except etiocholanolone and 11ß-hydroxy-etiocholanolone were significantly lower for the cyclists than for the sport students (Table 8.1), which may be due to the difference between physical workload (post competition versus resting conditions). But the sum of all steroid concentrations, which may be regarded as an integrating parameter of the steroidal biosynthesis, shows no significant difference.

USADA 0922

Ratios of some of the endogenous steroids are listed in Table 8.2. These values are used to indicate possible application of testosterone or epitestosterone. No significant differences were found for the ratios T/Epi, A/T, A/Epi and E/T. The significant difference in the ratio of the 11ß-hydroxy-steroids may be attributed to the different physical workload. Only assumptions can be made to explain the significant difference in the A/E ratio. Possibly the metabolism to androsterone is decreased following competition, or nutritional habits may have an influence [16].

Figures 1 and 2 compare the lower limits of the reference ranges of androsterone, etiocholanolone, epitestosterone and testosterone. The lower confidence intervals of etiocholanolone, epitestosterone and testosterone overlap whereas the confidence intervals for androsterone are different. It should be noted that the confidence intervals for the sport students are larger than those of the cyclists because of the small sample size.

The upper reference limits for the testosterone concentration and the T/Epi ratio of both populations are well overlapping and the T/Epi ratio is below the limit of 6 set by the IOC Medical Commission.

The differences in the reference limits of the 11ß-hydroxy steroids and the respective ratio OHA/OHE can be explained by the resting and post competition conditions under which the samples were taken.

Table 8.1: Comparison of endogenous steroid concentrations of the cyclists (n=482) with the sport students (n=105), t-Test for independent samples. n.s. = not significant (p>.05).

steroid concentrations	mean ng/ml Cyclists	mean ng/ml Students	t-Test p-level
androsterone (A)	2474	3051	< .001
etiocholanolone (E)	2272	2166	= n.s.
epitestosterone (EPI)	37	50	< .001
testosterone (T)	42	52	< .01
11ß-hydroxy-androsterone (OHA)	858	1098	< .01
11ß-hydroxy-etiocholanolone (OHE)	412	322	< .05
sum A+E+Epi+T+OHA+OHE	6092	6738	= n.s.

Table 8.2: Comparison of endogenous steroid ratios of the cyclists (n=482) with the sport students (n=105), t-Test for independent samples. n.s. = not significant (p>.05).

steroid ratios	mean ng/ml Cyclists	mean ng/ml Students	t-Test p-level
T/Epi	1.43	1.37	= n.s.
A/E	1.2	1.51	< .001
OHA/OHE	3.24	6.0	< .001
A/T	99	130	= n.s.
A/Epi	86	81	= n.s.
E/T	93	83	= n.s.
E/Epi	80	61	< .001

4.4 Conclusion

These results can be used as a guidence to establish the reference values of urinary excreted endogneous steroids in athletes. The values establish upper and lower limits of steroids which can be used in doping analysis as an indicator to detect an altered steroid profile possibly influenced by a previous application of anabolic steroids. These reference values can also be used by other laboratories; however, the accuracy and precision of quantification between the different laboratories must also be established.

USADA 0923

References

1. Donike, M., Bärwald, K.-R., Klostermann, K., Schänzer, W., and Zimmermann, J.
Nachweis von exogenem Testosteron
in: Sport: Leistung und Gesundheit. Edt.: H.Heck, W.Hollmann, H.Liesen, R.Rost, Deutscher Ärzte Verlag Köln, (1983) 293.

2. Zimmermann, J.
Untersuchungen zum Nachweis von exogenen Gaben von Testosteron
Thesis Deutsche Sporthochschule Köln, Hartung-Gorre Verlag Konstanz, 1986.

3. Adamietz, B.
Zum Problem der Applikation endogener Substanzen. Statistisches Verfahren zur Bestimmung von Normbereichen.
Thesis, Deutsche Sporthochschule Köln, 1984.

4. Donike, M., Adamietz, B., Opfermann, G., Schänzer, W., Zimmermann, J., Mandel, F.
Die Normbereiche für Testosteron- und Epitestosteron-Urinspiegel sowie des Testosteron-/Epitestosteron-Quotienten
in: I.-W.Franz, H.Mellerowicz, W.Noack (Hrsg.)
Training und Sport zur Prävention und Rehabilitation in der technisierten Umwelt
Springer Verlag Berlin Heidelberg (1985) 503-507.

5. Donike, M., Geyer, H., Schänzer, W. and Zimmermann, J.
Die Suppression der endogenen Androgenproduktion durch Methandienon
in: I.-W.Franz, H.Mellerowicz, W.Noack (Hrsg.)
Training und Sport zur Prävention und Rehabilitation in der technisierten Umwelt
Springer Verlag Berlin Heidelberg (1985) 508-512.

6. Donike, M., Geyer, H., Kraft, M., and Rauth, S.
Longterm Influence of Anabolic Steroid Misuse on the Steroid Profile.
in: P.Belotti, G.Benzi, A.Ljungqvist (eds.)
Official proceedings. IInd International Athletic Foundation World Symposium on Doping in Sport, Monte Carlo 1989
International Athletic Foundation, Monte Carlo (1990) 107-116.

7. Donike, M., Geyer, H., Gotzmann, A., Kraft, M., Mandel, F., Nolteernsting, E., Opfermann, G., Sigmund, G., Schänzer, W., Zimmermann, J.
Dope analysis
in: P.Belotti, G.Benzi, A.Ljungqvist (eds.)
Official proceedings. Ist International Athletic Foundation World Symposium on Doping in Sport, Florenz 1987
International Athletic Foundation, Florence (1988) 53-80.

8. Donike, M., Rauth, S., and Sample, B.
Excretion of ephedrine and endogenous steroids under conditions of controlled water intake and of water diuresis.
in: Proceedings of the 10th Cologne Workshop on Dope Analysis, in press.

8. Handbuch CSS: Statistica Volume I-III
Tulsa 1986-1991

9. International Federation of Clinical Chemistry (Expert Panel on Theory of Reference Values)
The theory of reference values. Part 5. Statistical treatment of collected reference values. Determination of reference limits.
J. Clin. Chem. Clin. Biochem. 25 (1987) 645-656.

10. Solberg, H.E.
"REFVAL"
Tech.Rep. Department of Clinical Chemistry, Rikshospitalet Oslo, 1983.

11. Solberg, H.E. and Grāsbeck, R.
Reference Values
Advances in Clinical Chemistry, Vol. 27. Academic Press, New York, 1989.

12. Grāsbeck, R. and Saris, N.E.
Establishment and use of normal values
Scand. J. Clin. Lab. Invest. 24 (Suppl. 110) (1969) 62.

13. Martin, H.F., Gudzinowicz, B.J., and Fanger, H.
Normal values in clinical chemistry
A guide to statistical analysis of laboratory data
Marcel Dekker , New York, 1975.

14. Reed, A.H.
Influence of Statistical Method Used on the Resulting Estimate of Normal Range
Clin.Chem. 17 (1971) 275-284.

15 Kappas, A., Anderson, K.E., Conney, A.H., Pantuck, E.J., Fishman, J., and Bradlow, H.L.
Nutrition-endocrine interactions: Induction of reciprocal changes in the Δ^{4}-5α-reduction of testosterone and the cytochrome P-450-dependen oxidation of estradiol by dietary macronutrients in man.
Proc.Natl.Acad.Sci. USA, 80 (1983) 7646-7649.

USADA 0924

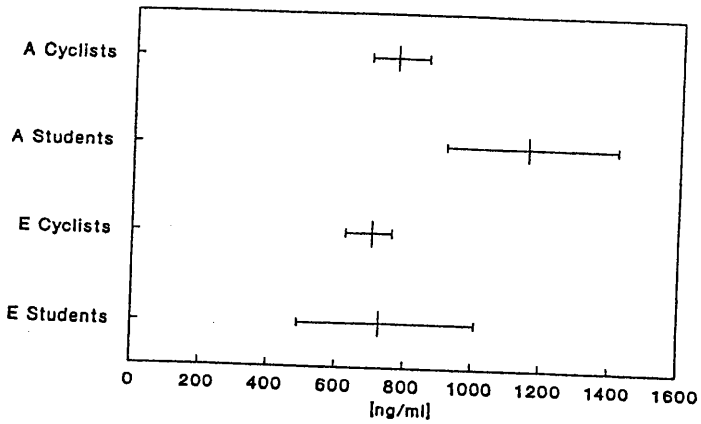


Figure 1: Lower reference limits of androsterone (A) and etiocholanolone (E), and lower confidence intervals: 90% for cyclists and 85% for sport students.
|—| confidence interval
| reference limit

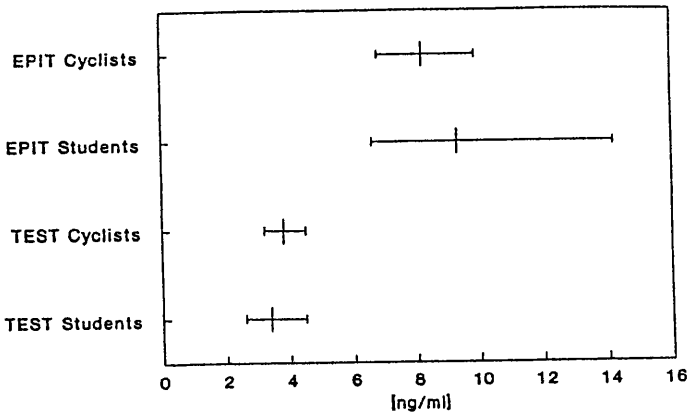


Figure 2: Lower reference limits of epitestosterone (EPIT) and testosterone (TEST), and lower confidence intervals: 90% for cyclists and 85% for sport students.
|—| confidence interval
| reference limit

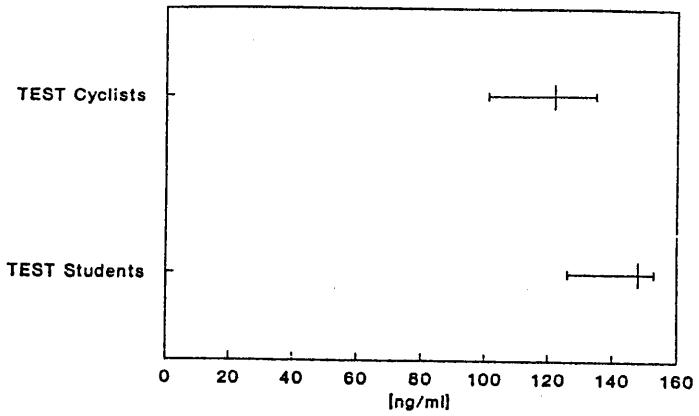


Figure 3: Upper reference limits of testosterone (TEST), and upper confidence intervals: 90% for cyclists and 85% for sport students.
|—| confidence interval
| reference limit

USADA 0925

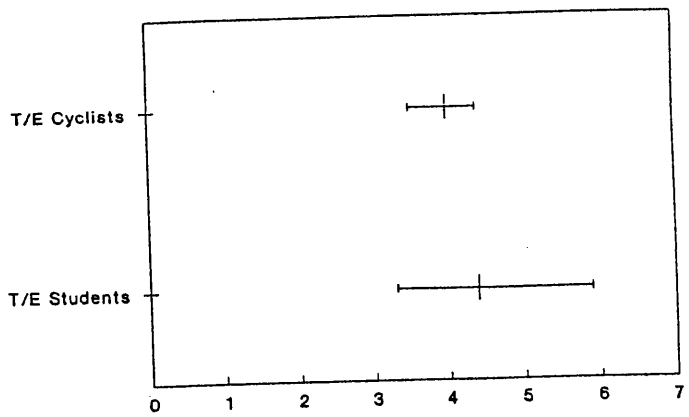


Figure 4: Upper reference limits of testosterone / epitestosterone ratio (T/E), and upper confidence intervals: 90% for cyclists and 85% for sport students.
|—| confidence interval
| reference limit

USADA 0926

U. Mareck-Engelke, H. Geyer and M. Donike

Stability of Steroid Profiles

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

In the present study the intraindividual variation i.e. the stability of ratios of endogenous steroids in male and female urine was investigated. In this study five male (age: $\bar{x}$ = 33±4,9 years) and five female subjects (age: $\bar{x}$ = 33±3,5 years) participated. Morning urine samples were collected for 30 days (men) respectively for one menstrual cycle (women). The urines were prepared according to the screening procedure of conjugated anabolic steroids and analyzed by GC/MS (1). The following steroid glucuronides were measured: androsterone (and), etiocholanolone (etio), testosterone (t), epitestosterone (epit), 11ß-OH-androsterone (OHa), 11ß-OH-etiocholanolone (OHe), 5α-androstane-3α,17ß-diol (a-diol) and 5ß-androstane-3α,17ß-diol (b-diol). The results of selected ratios of the endogenous steroids and their statistical evaluation are shown in Tables 1-4.

Following conclusions may be drawn:

- the steroid profile of men is more stable then that of women
- most stable parameter in all volunteers is the ratio androsterone/etiocholoanolone
- the ratio testosterone/epitestosterone shows also little variation in men
- the instability of the ratio testosterone/epitestosterone in women may be due to:
 a) concentrations are near by detection limit
 b) frequently other endogenous substances like 11-ketosteroids are coeluted with testosterone and epitestosterone (extraction of the urine by n-pentane removed a part of these substances)

References

(1) Donike M., Geyer H., Gotzmann A., Kraft M., Mandel F., Nolteernsting E., Opfermann G., Sigmund G., Schänzer W. and Zimmermann J.: Dope Analysis. In: Official Proceedings of the International Athletic Foundation World Symposium on Doping in Sport. P. Bellotti, G. Benzi, A. Ljungqvist (Hrsg.). IAAF Florence (1988) 53-87.

USADA 0927

Table 1: Stability of steroid profile (male urines): coefficient of variation (%) of some selected ratios (V1-V4 = Volunteer 1-4)

	V1	V2	V3	V4	V5
and/etio	10	9.8	6.5	11.2	10.6
t/epit	11.4	11	11	16.2	16
and/t	13.1	13.6	18	16.4	22
a-diol/b-diol	26	13.1	11.2	7.5	18.3
and/a-diol	15.2	18.6	16.1	16.2	24
and/b-diol	21	23	16.7	15.8	29
etio/a-diol	21	23	14.7	18.9	22
etio/b-diol	17.5	23	13.7	15.7	24
OHa/OHb	35	56	22	29	50

Table 2: Stability of steroid profile (male urines): statistics of some selected ratios (min = minimum value; max = maximum value; st. dev. = standard deviation; c.v. = coefficient of variation (%); V1-V4 = Volunteer 1-4)

	V1	V2	V3	V4	V5
and/etio					
min	1.24	1.00	0.93	1.08	1.07
max	1.99	1.42	1.20	1.75	1.76
mean	1.54	1.20	1.04	1.40	1.44
st.dev.	0.15	0.12	0.07	0.16	0.15
c.v.(%)	10	9.8	6.5	11.2	10.6
t/epit					
min	1.41	0.11	0.79	0.69	0.76
max	2.20	0.16	1.22	1.46	1.53
mean	1.82	0.13	0.91	0.90	1.04
st.dev.	0.21	0.01	0.10	0.15	0.17
c.v.(%)	11.4	11	11	16.2	16

Table 3: Stability of steroid profile (female urines): coefficient of variation (%) of some selected ratios (V1-V4 = Volunteer 1-4)

	V1	V2	V3	V4	V5
and/etio	12.4	10.6	12.3	15.9	15.3
t/epit	38.2	59	56	61	26.5
and/t	23.3	37.5	39.7	71	28.1
a-diol/b-diol	10.3	16.2	24.8	13.3	54
and/a-diol	18.4	29	34.9	10.9	18.2
and/b-diol	19.2	32.7	33.5	20.8	50
etio/a-diol	12.1	32.1	37.1	11.9	17.2
etio/b-diol	13	36.5	31.7	10.2	36.1
OHa/OHb	47	41.8	39.2	26.2	30.7

Table 4: Stability of steroid profile (female urines): statistics of some selected ratios (min = minimum value; max = maximum value; st. dev. = standard deviation; c.v. = coefficient of variation (%); V1-V4 = Volunteer 1-4)

	V1	V2	V3	V4	V5
and/etio					
min	0.83	1.09	0.59	0.54	0.51
max	1.29	1.84	0.99	0.95	0.94
mean	1.08	1.55	0.76	0.76	0.65
st.dev.	0.13	0.16	0.10	0.12	0.10
c.v.(%)	12.4	10.6	12.3	15.9	15.3
t/epit					
min	1.65	0.47	1.07	0.16	1.12
max	5.70	3.10	7.74	1.50	2.78
mean	3.07	1.07	2.35	0.65	1.71
st.dev.	1.17	0.63	1.31	0.40	0.45
c.v.(%)	38.2	59	56	61	26.5

USADA 0928

M.Donike, S. Rauth, U. Mareck-Engelke, H. Geyer and R. Nitschke

Evaluation of Longitudinal Studies, the Determination of Subject Based Reference Ranges of the Testosterone / Epitestosterone Ratio

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

The observations made in the past in measuring the steroid profile prove that for a given individual the excreted quantities of endogenous steroids are more or less constant [1]. The reason for this is the homeostasis of biosynthesis and metabolism of endogenous steroids [2, 3]. The concentrations of the eliminated endogenous steroids vary according to the urinary flow, whereas the concentration ratios of steroids are more constant [4].

The homeostasis of the endocrine steroid biosynthesis and metabolism is not disturbed by physical workload at least not to such an extend that the T/E ratio of an individual is altered [5]. The T/E ratios in longitudinal studies fluctuate around the mean with a CV which will not exceed 30%.In table 1 the T/E ratio of sport students with moderate physical activity are summarized. High work load will also not influence the T/E ratio to a greater extend. The T/E ratios of a professional cycling team participating in the Tour de France 1992 with 9 riders are presented in table 2. Training in high altitude had also no influence on the T/E ratio as we observed in following up the German rowing team before the 1988 Olympic Games in Seoul.

In case of testosterone application, the concentration of testosterone glucuronide will be increased more than any of the other testosterone metabolites leading to a significant increase of the individuals ratios of testosterone to epitestosterone [6, 7]. Depending on the route of administration, the dose, chemical structure of the ester etc. the T/E ratio will be elevated for some time before it returns to normal.

The increase of the T/E ratio is more prominent than the increase of other ratios within the steroid profile because testosterone is not metabolized to epitestosterone but contributes to androsterone and etiocholanolone [6, 7]. But nevertheless, also the ratios of testosterone to androsterone or etiocholanolone are increased and maybe used as additional parameters.

USADA 0929

To monitor testosterone application the question to answer is, if the ratio observed in an athlete's urine is higher than "normal".

Two possible approaches to answer this question are:
1. Comparison with a **population-based** reference range [8]
2. Comparison with the **subject-based** reference range.[8, 9]

The first approach has been chosen 1982 by the IOC Medical Commission to define a decision limit for testosterone application (T/E=6) [10, 11]. This ratio discriminates testosterone users from non-users only when the epitestosterone concentration is proportional to the concentrations of the other endogenous steroids. In case of a low epitestosterone concentration a few individuals may have a T/E ratio of higher than 6. This observation led to the decision of the IOC Medical Commission to recommend a follow-up of T/E ratios between 6 and 10 by longitudinal studies, to exclude physiological reasons for an elevated T/E ratio [12].

The proposal of the IOC Medical Commission to collect data in longitudinal investigations for an athlete found to have a T/E-ratio higher than 6 and lower than 10 provides the data to calculate a subject-based reference range [9].

The necessary data may be obtained:
1. by requesting from the authorities documented T/E ratios of previous tests - retrospective data
2. by additional unannounced in and/or out of competition doping controls - prospective data
3. a study under quarantaine-like conditions.

USADA 0930

Evaluation of the longitudinal approach

The problem can be statistically defined as the discrimination of an outlier from the series of T/E-ratios measured on the same individual. An outlier can be defined e.g. as a value lying outside the mean ±4 times the standard deviation calculated without the suspicious value.

For a consensus approach the following parameters must be defined:
1. the number of T/E ratios (in addition to the suspicious value)
2. the statistical probability
3. the time interval in which additional samples shall be collected
4. the acceptance of retrospective values
5. the definition of an outlier.

Proposal for the calculation of the subject-based reference range [9]:
1. calculate the mean out of at least 3 observations
2. calculate the limit for the outliers (l_o) following the formula below:

$$l_o = \text{mean} + t(p,n) * \text{stdev}$$

Note: All factors which may influence the analytical measurements will lead to a greater standard deviation, e.g. values determined in different laboratories or extent of physical workload. The greater standard deviation will give a higher limit for outliers and by this be in favor of the athlete.

Table 1: T/E-ratio of 5 male volunteers under resting conditions; urine samples taken in the morning [1].

cyclist	n	mean	s	c.v.%	min	max
1	30	1.82	0.21	11.4	1.41	2.20
2	30	0.13	0.01	11.0	0.11	0.16
3	30	0.91	0.10	11.0	0.79	1.22
4	30	0.90	0.15	16.2	0.69	1.46
5	30	1.04	0.17	16.0	0.76	1.53

Table 2a: T/E-ratio of 9 cyclists participating in the Tour de France 1992; urine samples collected over night.

cyclist	n	mean	s	c.v.%	min	max
1	9	1,81	0,34	18,82	1,36	2,45
2	14	0,22	0,06	27,69	0,10	0,34
3	23	2,02	0,15	7,47	1,72	2,38
5	22	1,85	0,24	13,02	1,36	2,44
6	11	0,12	0,04	38,00	0,09	0,24
7	21	1,22	0,16	12,67	0,78	1,48
8	15	0,84	0,08	9,24	0,69	0,96
9	21	1,87	0,34	18,24	1,28	2,55

Table 2b: T/E-ratio of 9 cyclists participating in the Tour de France 1992; urine samples taken after each stage.

cyclist	n	mean	s	c.v.%	min	max
1	7	2,34	0,28	12,11	1,90	2,72
2	11	0,24	0,08	31,88	0,10	0,35
3	18	2,34	0,36	15,25	1,56	2,78
5	21	2,38	0,59	24,80	1,21	3,55
6	9	0,13	0,02	19,08	0,10	0,18
7	18	1,32	0,21	15,82	0,95	1,60
8	12	0,76	0,14	18,25	0,62	1,07
9	19	1,80	0,32	17,77	1,36	2,61

USADA 0931

Table 3: T/E-ratio of the German rowing team (males) in a training camp in high altitude preparing the Olympic Summer Games in Seoul from the 18th August till the 7th September 1988.

Urine samples have collected over a time period of about 12 hours overnight.

rower	N	mean	stdev	vc %	min	max
1	7	1,99	0,14	7,09	1,76	2,11
2	6	4,04	0,53	13,21	3,20	4,81
3	6	0,87	0,20	23,41	0,68	1,24
4	5	0,93	0,14	14,99	0,80	1,17
5	5	2,79	0,30	10,88	2,42	3,10
6	6	0,08	0,02	24,79	0,06	0,11
7	7	1,06	0,22	20,81	0,81	1,38
8	5	1,39	0,17	12,36	1,20	1,63
9	4	1,79	0,40	22,55	1,40	2,27
10	6	2,63	0,57	21,50	1,59	3,25
11	6	0,72	0,07	9,32	0,63	0,82
12	0	no data	no data	no data		
13	5	0,74	0,07	9,18	0,63	0,79
14	7	0,51	0,13	26,29	0,34	0,67
15	7	0,93	0,19	20,68	0,68	1,23
16	6	0,20	0,03	13,87	0,17	0,24
17	7	1,77	0,29	16,38	1,27	2,10
18	6	3,93	0,97	24,77	2,92	5,38
19	7	0,76	0,16	20,69	0,52	1,00
20	6	0,84	0,14	16,56	0,70	1,08
21	6	0,17	0,04	20,93	0,13	0,22
22	7	1,15	0,19	16,48	0,86	1,43
23	7	0,81	0,24	29,61	0,50	1,11
24	6	0,10	0,05	52,47	0,06	0,20

References

1. MARECK-ENGELKE, U.; GEYER, H.; DONIKE, M.: Stability of Steroid Profiles. In: *10th Cologne Workshop on Dope Analysis, 7th to 12th June 1992 - Proceedings, M. Donike, H. Geyer, A. Gotzmann, U.Mareck-Engelke und S. Rauth (Hrsg.),* Sport und Buch Strauß, Köln (1993), 87-89
2. Shackleton, C.H.L.: Profiling steroid hormones and urinary steroids. *J. Chromatogr.* 379 (1986), 91
3. VESTERGAARD, P.: Excretion of common neutral steroids in healthy subjects as estimated by multi-column chromatography. In: *Estimation after multi-column liquid chromatography of common urinary neutral steroids with an application to the assay of plasma 17-oxosteroids. Vestergaard, P.; Sayegh, J.F.; Mowat, J.H.; Hemmingsen, L. (Hrsg.), Acta Endocrin.* 88 (1978), Suppl. 217, 157-189.
4. DONIKE, M.; RAUTH, S.; SAMPLE, B.: Excretion of ephedrine and endogenous steroids under conditions of controlled water intake and of water diuresis. In: *10th Cologne Workshop on Dope Analysis, 7th to 12 th June 1992 - Proceedings. Donike, M., H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (Hrsg.),* Sport und Buch Strauß, Köln (1993), 163-176
5. GEYER, G.: Die gas-chromatographisch/massenspektrometrische Bestimmung von Steroidprofilen im Urin von Athleten Deutsche Sporthochschule Köln: Dissertation, 1990
6. ZIMMERMANN, J.: *Untersuchungen zum Nachweis von exogenen Gaben von Testosteron* Hartung-Gorre Verlag, Konstanz, Diss. Deutsche Sporthochschule Köln, 1986
7. DONIKE, M.: Steroid Profiling in Cologne. In: *10th Cologne Workshop on Dope Analysis, 7th to 12th June 1992 - Proceedings. M. Donike, H. Geyer, A. Gotzmann, U.Mareck-Engelke und S. Rauth (Hrsg.),* Sport und Buch Strauß, Köln (1993), 47-86
8. SOLBERG, H.E.; GRÄSBECK, R.: *Reference Values* Bd. 27. Advances in Clinical Chemistry. Academic Press, New York, 1989
9. HARRIS, E.K.: Some Theory of Reference Values. I. Stratified (Categorized) Normal Ranges and a Method for Following an Individual's Clinical Laboratory Values. *Clin.Chem.* 21 (1975), 10, 1457-1464
10. ADAMIETZ, B.: *Zum Problem der Applikation endogener Substanzen. Statistisches Verfahren zur Bestimmung von Normbereichen.* Deutsche Sporthochschule Köln: Examensarbeit, 1984
11. DONIKE, M.; ADAMIETZ, B.; OPFERMANN, G.; SCHÄNZER, W.; ZIMMERMANN, J.; MANDEL, F.: Die Normbereiche für Testosteron- und Epitestosteron-Urinspiegel sowie des Testosteron-/Epitestosteron-Quotienten. In: *Training und Sport zur Prävention und Rehabilitation in der technisierten Umwelt. I.-W.Franz, H.Mellerowicz, W.Noack (Hrsg.),* Springer Verlag, Berlin, Heidelberg (1985), 503-507
12. INTERNATIONAL OLYMPIC COMMITTEE, Medical COMMISSION: List of Doping Classes and Methods. 17th March 1993.

USADA 0932

J. Baenziger and L. Bowers

Variability of T/E Ratios in Athletes

Sports Medicine Drug Identification Laboratory, Department of Pathology and Laboratory Medicine, Indiana University Medical Center, Indianapolis, Indiana, USA

INTRODUCTION:

The use of the Testosterone(T)/Epitestosterone(E) Ratio (T/E) has been the primary method used to determine whether an athlete has used exogenous testosterone. The finding of T/E results greater than 6:1 has been considered as a positive anti-doping test by the IOC since 1982. Since this time a few athletes were identified which had elevated T/E ratios and after extensive evaluation this elevated T/E ratio was considered to be due to a normal physiologic variation. Many athletic programs will now more frequently test those individuals who have a T/E ratio between 6-10:1 before considering taking punitive actions[1]. However, it is also known that some athletes are using exogenous testosterone or other manipulations of the endocrine system by various substances, yet their T/E ratios never exceed 6:1. Several methods are being investigated to help resolve the above problems[2], and we now present preliminary data on the use of T/E Ratio variability as a tool to help resolve some of these problems.

SUBJECTS:

Urine samples were collected from a group of male athletes (subjects) as part of their athletic organization's Anti-doping Program during 1990-1991. Multiple samples from the subjects were received over this time period and were uniquely identified by a sample number so that the analyses of the T/E ratios were performed blind to the relationship between samples. The actual identity of the subject is only known to the athletic organization with each subject having a unique player number assigned. Subsequent to the analysis, the athletic organization generated a list of sample numbers that were associated

USADA 0933

with the unique player number for our use in matching sample results for each subject.

METHODS:

Anabolic Steroid Analysis:

The T/E Ratio was determined on each subject's sample(s) using our laboratory's routine procedure for conjugated steroids[3]. In brief, 5 ml of urine is extracted using a C-18 solid phase column. The column is eluted with methanol, evaporated, and then hydrolyzed using Helix pomatia. The hydrolysate is extracted with ether and then derivitized with MSTFA/TMIS to silinate all hydroxyl and keto functions. Analysis is performed using one of 5 Hewlett/Packard 5890 GC's equipped with a capillary column and attached to 5970B MSD detectors operated in the SIM mode. The T/E ratio's were determined by comparing the peak area of testosterone and epitestosterone using the $M^{+\bullet}$ (432 m/z) ion signal. Additional analytical data from endogenous urinary steroids is also routinely collected and recorded along with other sample data as follows:

Specimen Data	Analytical Data
Subject's specimen ID#	Androsterone(A) & Etiocholanolone(Et) areas
Collection date of specimen	11-ßOH A & Et areas
Receipt date of specimen	Testosterone & Epitestosterone areas
Subject's sport	Internal Standard area
Subject's sex	Creatinine concentration
Laboratory Accession #	Specific gravity
Laboratory Batch #	Urine pH

Within each analytical batch analysis (20 subject samples) the following controls and standards are simultaneously analyzed: a conjugated control (both abnormal and normal); plus an endogenous standard which includes A, Et, 11-OH A, 11-OH Et, T, and E. Thus, semi-quantitative concentrations for the endogenous steroids can be determined on each subject by comparing area ratios for each steroid with the endogenous standard. These concentrations are currently being entered into a comprehensive subject database.

Data Evaluation:

The results of the T/E ratio determinations for each subject were evaluated and the mean, standard error of the mean (SEM), standard deviation (SD), percent coefficient of variation (%CV), and percent standard error of the mean (%SEM) were calculated using routine statistical methods. A subset of subjects were selected for further evaluation of T/E variability and consisted of those subjects who had at least one T/E result greater than 3 and who had at least 3 samples analyzed.

RESULTS:

Analytical Performance:

The analytical precision of the T/E ratio for the screening procedure has been determined by recording the T/E ratio from both the abnormal (mean = 7.4, %CV = 8) and normal (mean = 1.4, %CV = 15) conjugated controls over multiple batch assays and across 5 instruments.

Subject Population Characteristics:

A total of 1285 subjects were tested with 4973 T/E results measured. During this same time period a total of 22,806 T/E ratios were performed on athletes from all sports in the laboratory. The two populations' T/E ratios are compared in Table 1 and Figures 1 and 2, which show that the two populations are similar.

The mean T/E was determined in a subset of 796 subjects who had at least 3 values recorded and compared to the mean of the 1285 subjects. These populations' T/E ratios are compared in Table 1 and in Figures 3 and 4, which again show that the populations are similar. The populations are further characterized by determining the values at the 90th, 95th, and 99th percentiles for these non-gaussian population distributions.

Subject Variability of T/E Ratios:

The variability of each subject's T/E determinations were calculated on the population subset of 796 subjects who had 3 or more values recorded. Figure 5 shows the distribution of the number of values that have been recorded for each subject. The results of these variability determinations are summarized in Tables 2-3 and in Figures 6-9. For each variability statistic a plot of the distribution frequency as well as a plot versus the mean T/E ratio was made. The variability determinations demonstrated a non-gaussian distribution and so the 90th, 95th, and 99th percentile values are listed in the Tables.

Subject Screening Procedure:

A second subset of the total 1285 subject population was selected on the basis of

USADA 0934

whether a subject had at least one T/E result > 3:1 (approximately the 90th percentile for all athletes). This subset was further evaluated to determine the variability in their T/E results. A total of 122 subjects with 629 T/E measurements were identified using the above criteria. Of these 122 subjects, 97 subjects (8% of total subjects) also had at least 3 T/E values to use for variability calculations. These 97 subjects could be divided into three basic patterns of T/E variability:

Pattern Type 1: elevated T/E ratio but with low variability

Pattern Type 2: high variability as the result of a T/E "spike"

Pattern Type 3: high variability without a distinct T/E "spike"

Of these 97 subjects, 33 subjects had Pattern Type 1, 38 subjects had Pattern Type 2, 21 subjects had Pattern Type 3, and 5 subjects could not be classified into either Pattern 2 or 3. Graphical representations of a selected number of each Pattern Type are shown in Figures 10-12.

DISCUSSION/CONCLUSIONS:

These preliminary results of the variability of T/E ratios on this population of athlete subjects shows that the vast majority of subjects have low T/E ratios (ie clustered around the population mean of 1.2) and demonstrate little variability in their T/E ratios over time (ie %CV < 30%). Comparing the T/E variability (%CV) between the subjects and the analytical precision it is concluded that most of a subject's T/E variability is a determinate of the analytical imprecision. However, a small fraction of the subjects do show large variability in their T/E measurements over time and thus warrant additional evaluation.

The finding of a small fraction of subjects with relatively high T/E ratios (ie > 3:1) and with low variability (ie Pattern Type 1) may be due to a normal physiologic variation between subjects. In fact, our laboratory has documented several individuals who have had T/E ratios > 6:1 and who have demonstrated relatively consistent T/E ratios over several years and who have been further investigated using serum hormonal studies to document that the pituitary-testicular endocrine system is not suppressed or abnormal. The specific explanation for why T/E ratios may be elevated normally in some individuals is still not available, but research is currently underway that may help determine how this finding relates to an individual's production or metabolism of androgenic steroids.

USADA 0935

The use by athletes of aqueous based testosterone with its relatively short half-life, epitestosterone use, and other techniques that alter the endocrine system makes timing of specimen collection critical to detect an elevated T/E ratio on a single determination. To detect this type of anabolic steroid misuse it has been suggested that the T/E ratio cut-off be lowered (ie 3:1) and that serial specimens be collected and used to evaluate the variability of an athlete's T/E measurements. The data presented in this study suggests that this may indeed be a viable method. These results indicate that the finding of an elevated T/E ratio (ie > 3:1) and a large T/E variability (ie > 30 %CV) is statistically uncommon in an athletic population. Thus those subjects with Pattern Type 3 are of interest since the large variability in T/E ratio results indicates variable excretion of T and E which has been seen in persons who are receiving exogenous aqueous testosterone or in persons whose pituitary-testicular endocrine system has been variably suppressed by anabolic steroids[4,5]. In addition, the finding of an isolated "T/E spike" as is seen in the Pattern Type 2 cases is strong evidence that exogenous testosterone use has occurred.

FUTURE PLANS:

Investigations are underway in various laboratories that are evaluating the endocrine and metabolic changes that result from the use of exogenous testosterone or other synthetic anabolic steroids. Donike[6] has described the use of urine "steroid profiles" to detect previous exogenous testosterone or anabolic steroid use and consists of comparing the relative quantities of various androgenic steroid metabolites in urine. Donike has found that the excretion of various androgenic steroids is altered in individuals who have used synthetic anabolic steroids or exogenous testosterone. This current study is presented as a preliminary evaluation of the intra-individual variability of T/E ratios in a large population. All data from these subjects are stored on tape and subsequent evaluation will consist of the determination of the quantitative levels of testosterone, epitestosterone, and other endogenous androgenic steroids present in each sample. Thus, a combined evaluation of both the "steroid profile" and T/E variability over time will be investigated, and may be of additional benefit for the detection of anabolic steroid doping. Additional samples from these subjects are continually being analyzed and thus future decoding of these specimens will allow for this database to be continuously evaluated over time.

Additional measurements of urinary luteinizing hormone (LH) levels, plus serum hormone determinations when available will also be incorporated into the on-going study of these subjects.

REFERENCES:

1. International Olympic Committee, List of Banned Substances, 1993.

2. Palonek E and Garle M. "Single Injection of Testosterone to 7 Volunteers: Results from this Study". Proceedings of 10th Cologne Workshop on Dope Analysis, June, 1992. ISBN 3-89001-011-3

3. Sample, RHB and Baenziger, JC. Gas Chromatography/Mass Spectrometric Detection of Anabolic Steroids. In, Analytical Aspects of Toxicology, edited by Deutsch, John R. Wiley and Sons, Inc., New York, 1989.

4. Donike M, Geyer H, Schanzer W, and Zimmerman J. "Die Suppression der endogenen Androgenproduktion durch Metandienon" in Training and Sport zur Pravention und Rehabilitation in der technisierten Umwelt Eds. I.-W. Franz, H. Mellerowicz, W. Noack. Springer Verlag Berlin-Heidelberg-New York-Tokyo. 1985.

5. Donike M, Geyer H, Kraft M and Rauth S. "Longterm Influence of Anabolic Steroid Misuse on the Steroid Profile" in Official Proceedings of the 2nd International Athletic Foundation World Symposium on Doping in Sport, Monte Carlo, 1990.

6. Donike M. "Steroid Profiling in Cologne". in Proceedings of the 10th Cologne Workshop on Dope Analysis, Cologne, Germany, 1992. ISBN 3-89001-011-3

USADA 0936

Figure 1: T/E ratios from all athletes' samples received during 1990-91.

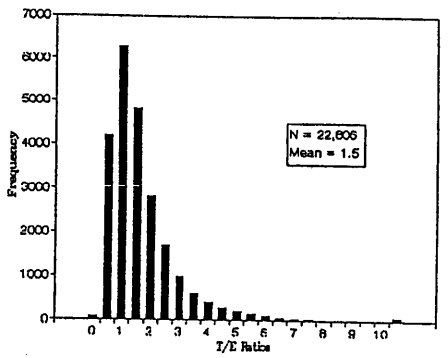


Figure 2: Distribution of T/E ratios from 796 subject athletes (who had 3 or more T/E values) received during 1990-91, and associated with 4,310 individual results.

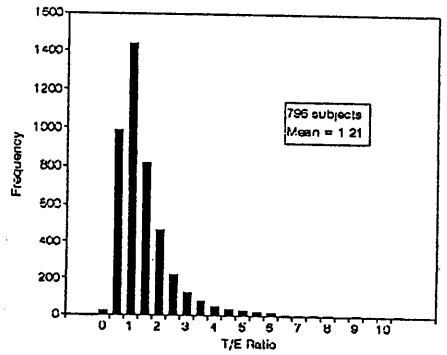


Table 1: Percentile cut-off levels for T/E ratios from the populations of all athletes, subjects from this study, and subsets of study subjects.

Statistic/Percentile Cut-off	#	90%	95%	99%
T/E's of all athletes 1990-91	22806	3-3.5		6.5-7
T/E's of 1285 subjects	4973	2.3	3.2	5.2
T/E Mean's of subjects	1285	2.2	2.9	5.0
T/E Mean's of subjects with 3 or > values	796	2.2	2.9	4.3

Figure 3: Distribution of T/E Means from the 1285 subjects.

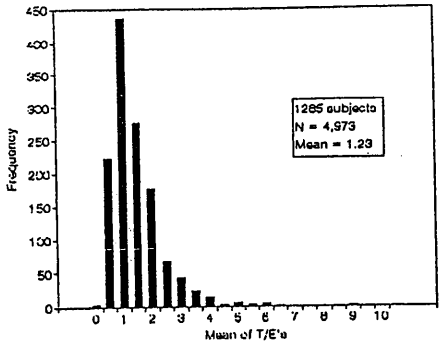


Figure 4: Distribution of T/E Means from those subjects with at least 3 values.

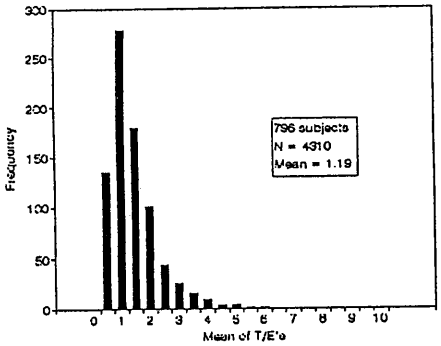
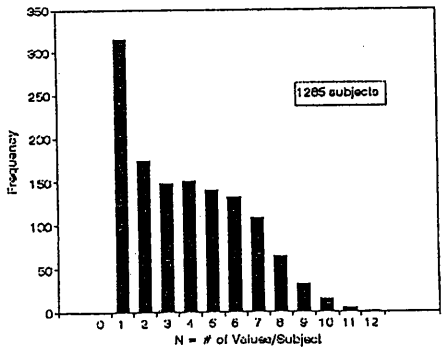


Figure 5: Distribution of the number of T/E values on the 1285 subjects.



USADA 0937

Figure 6: Distribution of subjects' standard deviations of T/E ratios.

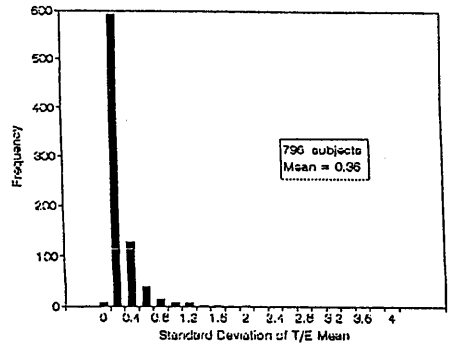


Figure 7: Comparison of subjects' T/E mean versus the standard deviation of the T/E measurements.

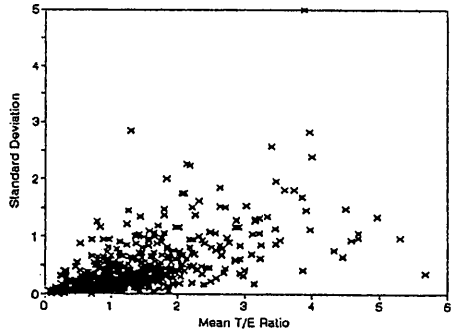


Table 2: Percentile cut-off levels for the standard deviation (SD) determinations grouped by T/E (mean) ranges.

Statistic/Percentile Cut-off	#	90%	95%	99%
SD of T/E's 0-1	405	0.38	0.52	0.95
SD of T/E's 1-2	284	0.81	1.01	1.45
SD of T/E's 2-3	68	1.5	1.76	2.24
SD of T/E's > 3	35	1.96	2.16	2.8
SD of T/E's of all subjects	796	0.87	1.16	1.96

Figure 8: Distribution of Percent Coefficient of Variation of subjects' T/E measurements.

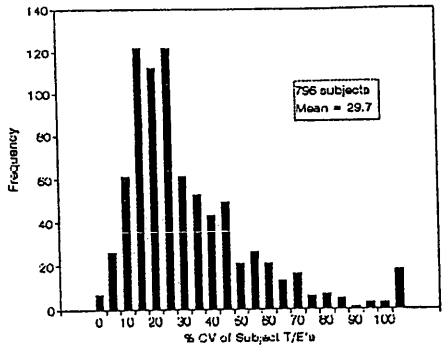


Figure 9: Comparison between the Percent Coefficient of Variation (%CV) of the subjects' T/E measurements versus the subject's mean T/E.

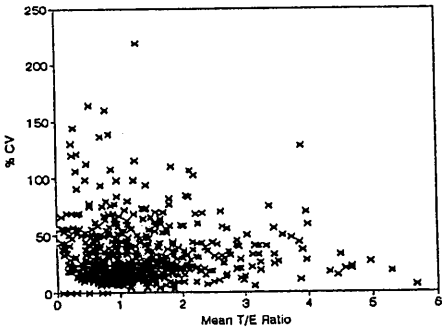


Table 4: Percentile cut-off level for the Percent Coefficient of Variation (%CV) of the subjects' T/E measurements grouped by T/E (mean) ranges.

Statistic/Percentile Cut-off	#	90%	95%	99%
%CV of T/E's 0-1	405	57	75	136
%CV of T/E's 1-2	284	55	68	98
%CV of T/E's 2-3	68	58	70	102
%CV of T/E's > 3	35	56	71	75
%CV of T/E's of all subjects	796	58	72	122

USADA 0938

Figure 10: Type 1 pattern of T/E variability (33 of 97 subjects) examples showing low variability; ie %CV < 30, and T/E > 3. (A = 6 %CV; B = 20 %CV; C = 19 %CV)

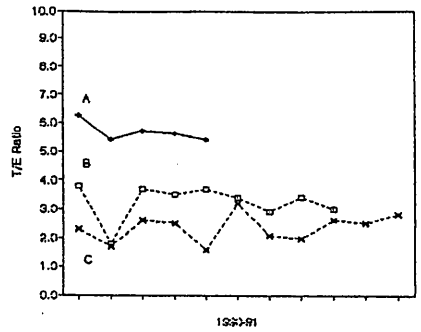


Figure 11: Type 2 pattern of T/E variability (38 of 97 subjects) examples showing a "spike" change in an athlete's T/E ratios.

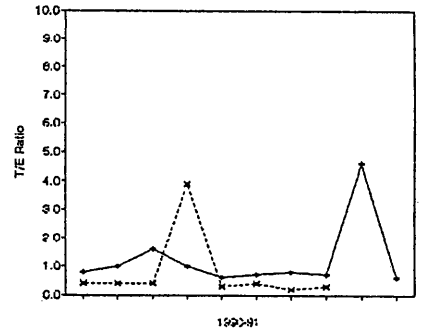
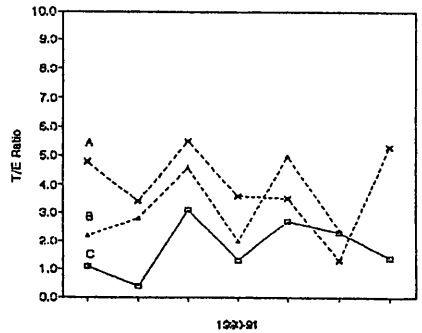


Figure 12: Type 3 pattern of T/E variability (21 of 97 subjects) examples showing a high degree of variability; ie %CV > 30 and T/E above 3. (A = 37 %CV; B = 55 %CV; C = 41 %CV)



U.Mareck-Engelke, H.Geyer and M.Donike

Stability of Steroid Profiles (3): The Circadian Rhythm of Urinary Ratios and Excretion Rates of Endogenous Steroids in Male

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

The stability of ratios and excretion rates of endogenous steroids in male urines was investigated. Five male (age: x=32±6 years) volunteers participated. Urine samples were collected every two hours (including during the night; altogether 12 samples) for 24 hours. The urines were prepared according to the screening procedure of conjugated anabolic steroids and analysed by GC/MS (1).

The following steroid glucuronides were measured and quantified: androsterone (A), etiocholanolone (E), testosterone (T), epitestosterone (epiT), 11ß-OH-androsterone (OHA), 11ß-OH-etiocholanolone (OHE), 5α-androstan-3α,17ß-diol (Adiol) , 5ß-androstan-3α,17ß-diol (Bdiol), pregnandiol (Pregnd) and tetrahydrocortisol (THF).

The results of selected steroid concentration ratios, their excretion rates and statistical evaluations are shown in Tables 1-4 and Figures 1-12.

USADA 0939

References

(1) Donike M., Geyer H., Gotzmann A., Kraft M., Mandel F., Nolteernsting E., Opfermann G., Sigmung G., Schänzer W. and Zimmermann J.: Dope Analysis
In: Official Proceedings of the International Athletic Foundation World Symposium on Doping in Sport. P.Bellotti, G.Benzi, A.Ljungquist (Hrsg.) IAAF Florence (1988) 53-87.

(2) Mareck-Engelke U., Geyer H. and Donike M.: Stability of steroid profiles
In: 10th Cologne Workshop On Dope Analysis, 7th To 12th June 1992 -Proceedings-, 87-89

(3) Mareck-Engelke U., Geyer H. and Donike M.: Stability of steroid profiles (2) : Excretion rates from morning urines In: Recent Advances In Doping Analysis
Proceedings of the11th Cologne Workshop On Dope Analysis, 7th to 12th March 1993 ,85-89

Conclusions

The ratio A/E is the most stable parameter (except for one volunteer). The variation is less than 10% (tab1,2 ; fig 1,2).
The ratios T/epiT, A/T, A/epiT and Adiol/Bdiol show coefficients of variation less than 30% for all volunteers.

The excretion rates of the endogenous steroids show strong intraindividual and interindividual variation (tab 3,4).
Maximum excretion rates were observed at daytime hours, minima at nighttime hours.
Maxima were stronger marked than minima.
Calculated were differences between excretion rates of all quantified steroids (max. and min.) and their corresponding mean values.
The calculated values for all steroids from all volunteers are presented in tab 5 and 6.
They are situated in a range characterised as: " mean ± 2.8 * st.dev."
These data are based on 12 data points per person and steroid.
The boarderlines of the subject-based reference ranges for persons under resting conditions within 24 hours (for n=12) may be defined as: L_o = mean $\pm$ 3.0 * st.dev.
The calculation factor t depends on the number of analysed and calculated samples from one individual. Decrease of sample numbers will lead to an increase of the calculation factor.

The ratio OHA/OHE decreases significantly during the night (fig.3).
The excretion of OHA and THF decreases significantly during the night (fig 4,5).
This circadianic character depends on the rhythm of ACTH. For all other steroids no common circadian rhythm is recognizable.

Between hormones which represent testicular activity (T, epiT) and those which represent adrenal activity (A,E) it was not possible to detect any difference referring to circadianic rhythm (fig 6-8).

For steroids with small variation in excretion during 24 hours (A,E,T,epiT) a small urine collecting interval (only a few hours or a morning urine) is representative for a 24 hour collected urine.
This is not possible for steroids with a large variation in excretion during 24 hours (steroids following the ACTH-rhythm: THF, OHA) (fig 9,10).

There is no correlation between excretion of endogenous steroids and flow of urine (fig 11,12).

USADA 0940

Table 1: Stability of steroid profiles (male urines).
Coefficient of variation (%) of some selected steroid concentration ratios
V1-V5 = volunteer 1-5

	V1	V2	V3	V4	V5
A/E	8	8	8	9	26
T/epiT	21	18	25	9	12
A/T	30	18	26	14	15
A/epiT	16	22	15	12	13
Adiol/Bdiol	21	14	7	6	20
A/Adiol	24	7	34	11	32
A/Bdiol	17	16	30	10	35
E/Adiol	23	11	32	14	18
E/Bdiol	15	14	28	11	13
OHA/OHE	48	61	56	51	67
A/THF	25	49	66	50	48

Table 2: Stability of steroid profiles (male urines).
Statistics of some selected steroid concentration ratios
min = minimum value max = maximum value
st.de. = standard deviation c.v. = coefficient of variation (%)
V1-V5 = volunteer 1-5

	V1	V2	V3	V4	V5
A/E					
min	1.2	0.7	1.0	1.6	0.9
max	1.6	0.9	1.3	2.3	2.5
mean	1.4	0.8	1.1	2.0	1.5
st.dev.	0.11	0.06	0.09	0.17	0.38
c.v. (%)	8	8	8	8	26

	V1	V2	V3	V4	V5
T/epiT					
min	1.2	0.11	0.7	4.8	0.8
max	2.5	0.23	1.3	6.6	1.3
mean	1.8	0.15	0.9	5.4	1.0
st.dev.	0.36	0.03	0.23	0.49	0.11
c.v. (%)	21	18	25	9	12

USADA 0941

Table 3: Stability of steroid profiles (male urines).
Coefficient of variation (%) of excretion rates
V1-V5 = volunteer 1-5

	V1	V2	V3	V4	V5
A	26	19	34	26	26
E	30	17	37	29	22
epiT	23	29	42	25	18
T	24	28	37	25	21
Adiol	23	22	37	25	26
Bdiol	31	17	34	26	25
OHA	48	46	46	52	50
OHE	40	30	48	56	56
THF	45	47	59	58	56

Table 4: Stability of steroid profiles (male urines).

Statistics of some selected excretion rates

min = minimum value max = maximum value

st.de. = standard deviation c.v. = coefficient of variation (%)

V1-V5 = volunteer 1-5

		V1	V2	V3	V4	V5
A (µg/h)	min	64	49	26	117	120
	max	136	97	153	258	288
	mean	85	71	99	169	189
	st.dev.	22	19	34	26	49
	c.v. (%)	26	19	34	26	26
E(µg/h)	min	45	68	23	55	82
	max	110	119	153	145	175
	mean	62	90	89	85	140
	st.dev.	18	15	33	25	31
	c.v. (%)	30	17	37	29	22
epiT (µg/h)	min	1.0	0.9	0.39	0.45	1.29
	max	2.2	2.2	3.0	1.1	2.38
	mean	1.6	1.6	1.6	0.76	1.71
	st.dev.	0.36	0.45	0.68	0.19	0.31
	c.v. (%)	23	29	42	25	18
T (µg/h)	min	1.4	0.14	0.52	2.4	1.08
	max	3.7	0.38	2.4	6.2	2.33
	mean	2.8	0.24	1.4	4.1	1.69
	st.dev.	0.65	0.07	0.51	1.01	0.35
	c.v. (%)	24	28	37	25	21

USADA 0942

Table 5: Calculation factor (t) for the upper boarderline (u_o) of the subject-based reference range following the formula: u_o = mean + t * st.dev.

V1-V5 = volunteer 1-5

	V1	V2	V3	V4	V5
A	2.4	1.9	1.7	2.0	2.0
E	2.6	2.0	1.9	2.4	1.1
epiT	1.8	1.5	2.0	2.0	2.2
T	1.5	2.1	1.8	2.1	1.8
Adiol	2.1	1.9	1.8	1.6	1.6
Bdiol	2.7	1.8	1.5	1.9	1.3
11OHA	2.2	1.5	1.7	2.3	1.8
11OHE	2.3	1.3	1.9	2.6	2.0
Pregnd	2.6	1.3	1.6	1.8	1.2
THF	1.7	1.7	1.6	2.0	1.5
mean	2.2	1.7	1.7	2.1	1.7

Table 6: Calculation factor (t) for the lower boarderline (l_o) of the subject-based reference range following the formula: l_o = mean + t * st.dev.

V1-V5 = volunteer 1-5

	V1	V2	V3	V4	V5
A	1.0	1.6	2.1	1.2	1.4
E	0.9	1.4	1.9	1.2	1.9
epiT	1.5	1.5	1.7	1.6	1.4
T	2.1	1.5	1.7	1.7	1.7
Adiol	1.2	1.2	1.3	1.7	2.4
Bdiol	1.0	1.7	1.5	1.7	2.0
11OHA	1.3	1.5	1.8	1.1	1.4
11OHE	1.4	1.3	1.3	1.1	1.0
Pregnd	1.3	1.7	2.0	1.3	2.0
THF	1.1	1.5	1.5	1.2	1.3
mean	1.3	1.5	1.7	1.4	1.7

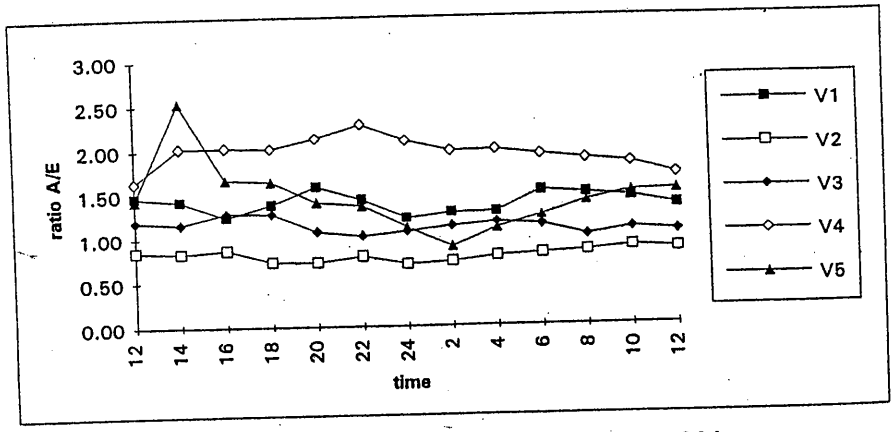


Fig 1: ratio (concentration) A/E in male urine, collected over 24 hours
V1-V5 = Volunteer 1-5

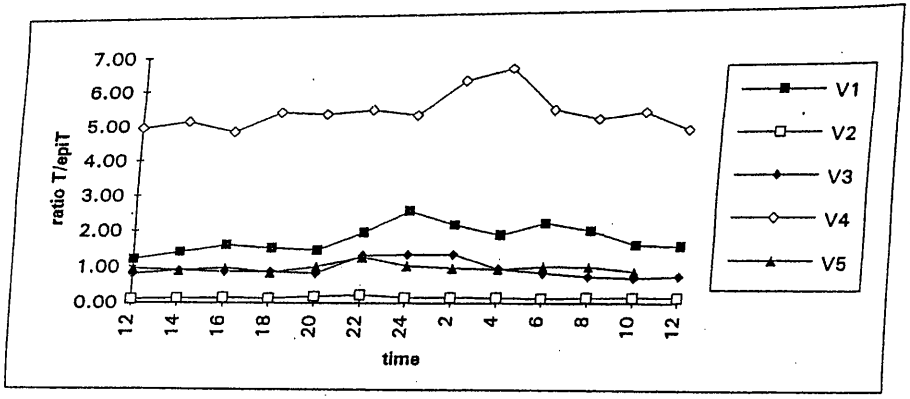


Fig 2: ratio (area) T/epiT in male urine, collected over 24 hours
V1-V5 = Volunteer 1-5

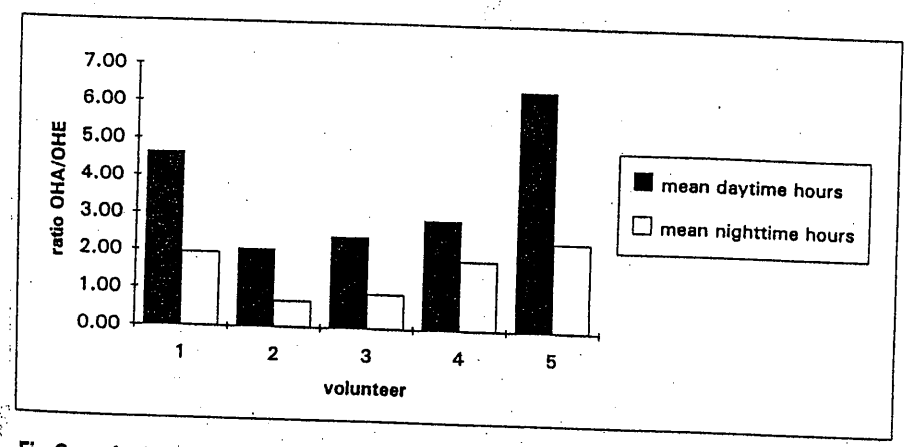


Fig 3: ratio (concentration) OHA/OHE in male urine, collected over 24 hours
V1-V5 = volunteer 1-5

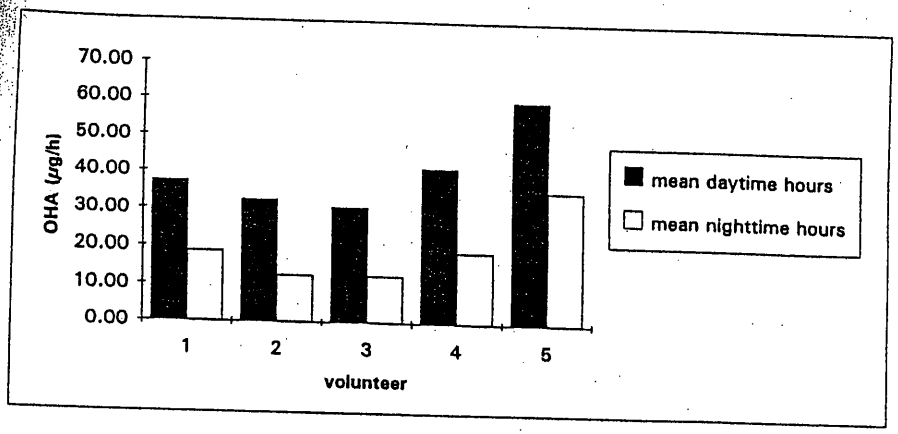


Fig 4: excretion OHA (µg/h) in male urine, collected over 24 hours
V1-V5 = volunteer 1-5

USADA 0943

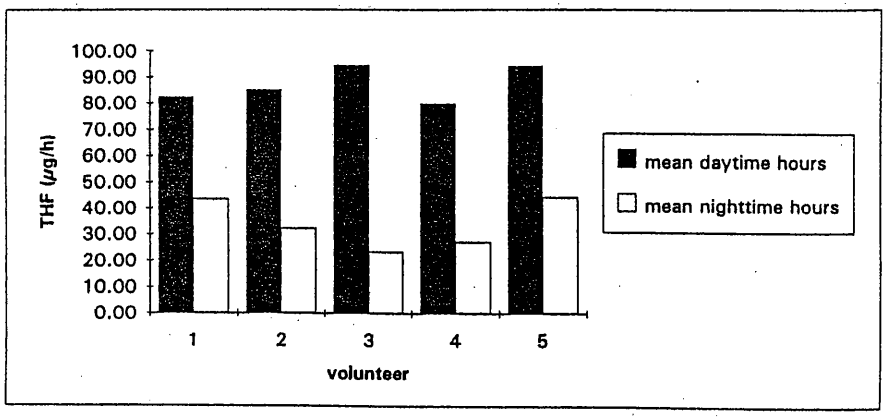


Fig 5: excretion THF (µg/h) in male urine, collected over 24 hours
V1-V5 = volunteer 1-5

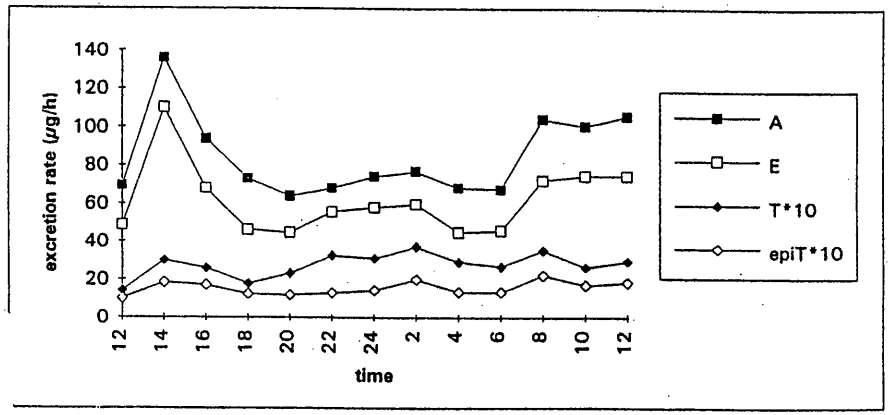


Fig 6: excretion rates of endogenous steroids in male urine,
collected over 24 hours
volunteer 1

USADA 0944

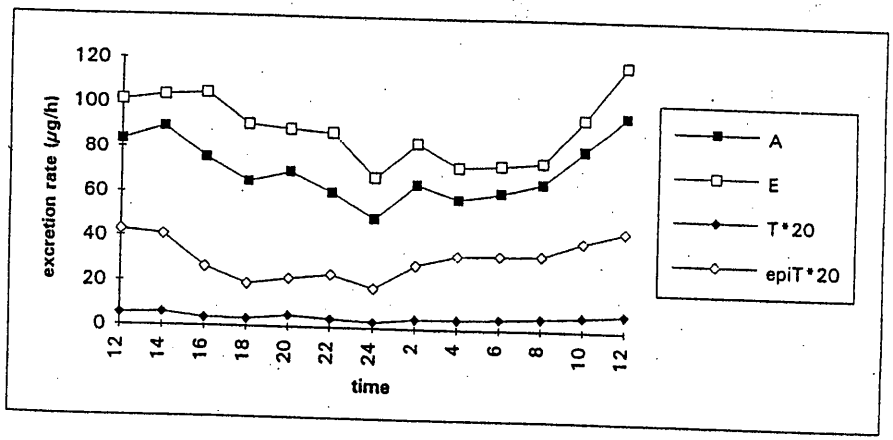


Fig 7: excretion rates of endogenous steroids in male urine,
collected over 24 hours
volunteer 2

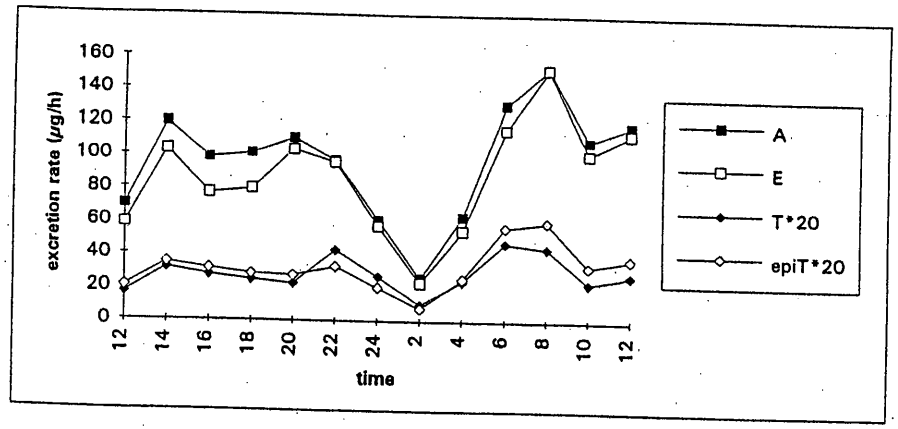


Fig 8: excretion rates of endogenous steroids in male urine,
collected over 24 hours
volunteer 3

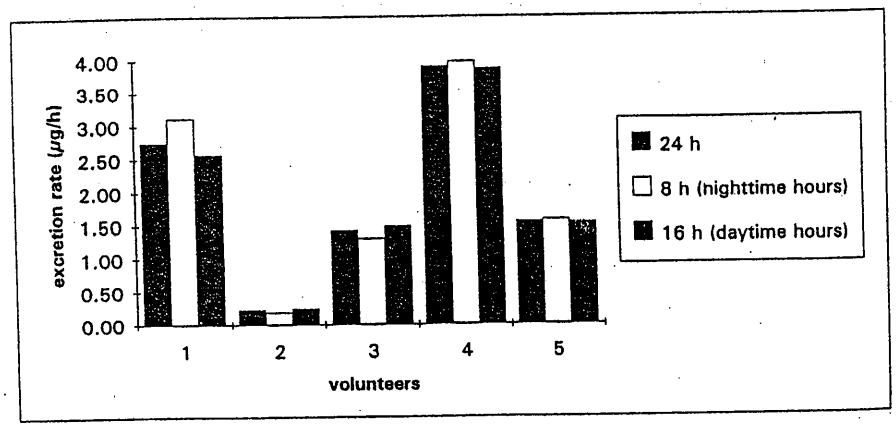


Fig 9: Testosterone excretion (µg/h)
comparison of daytime hours and nighttime hours with 24 hours collected urine
V1-V5 = volunteer 1-5

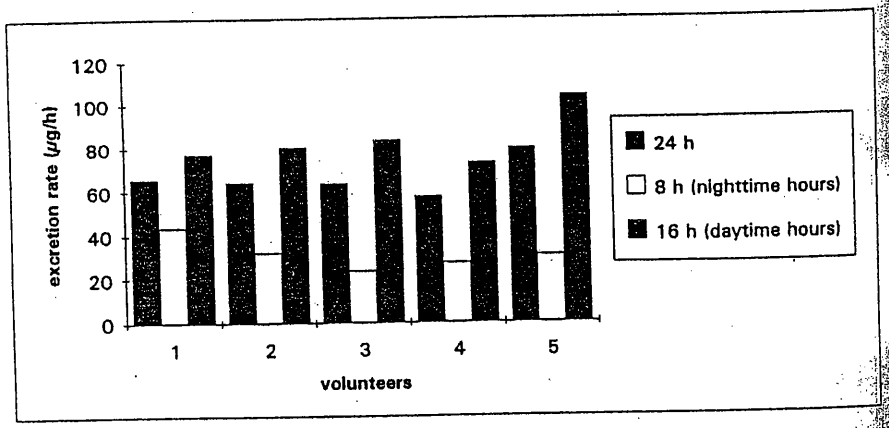


Fig 10: THF excretion (µg/h)
comparison of daytime hours and nighttime hours with 24 hours collected urine
V1-V5 = volunteer 1-5

The real calculated excretion amount for 24 hours is compared with values calculated for 24 hours from sleeping hours and wake hours.
Excretion rates obtained during the nighttime hours and daytime hours are added cumulatively and the values normalized to 24 hours.

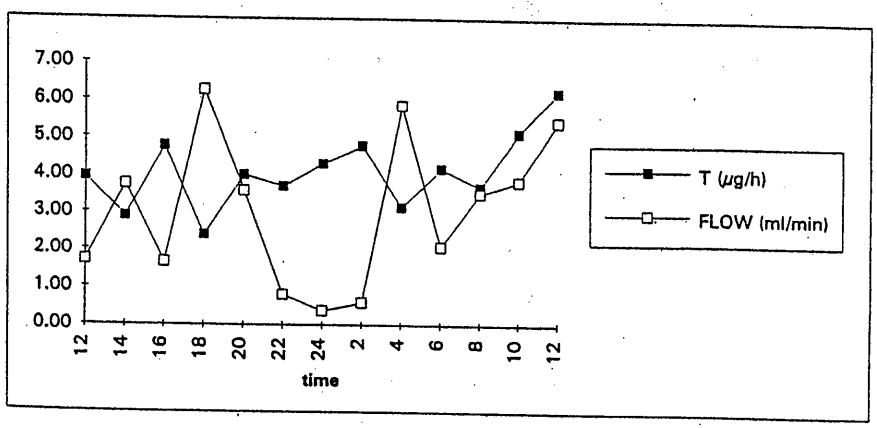


Fig 11: correlation between excretion of T (µg/h) and flow (ml/min)
Volunteer 4

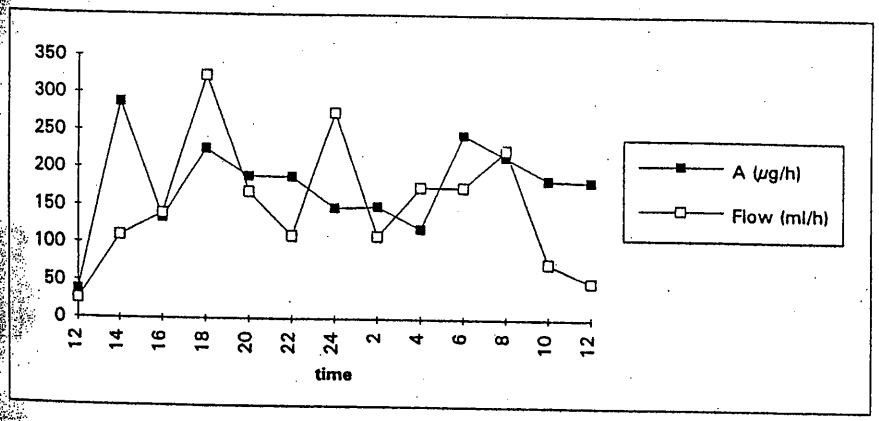


Fig 12: correlation between excretion of A (µg/h) and flow (ml/h)
Volunteer 5

USADA 0945

M. Donike, U. Mareck-Engelke and S. Rauth

STATISTICAL EVALUATION OF LONGITUDINAL STUDIES, PART 2: THE USEFULNESS OF SUBJECT BASED REFERENCE RANGES IN STEROID PROFILING

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

ABSTRACT

The results presented in this paper demonstrate that the subject-based reference ranges react more sensitive to variations than the population-based reference ranges, so that subject-based reference ranges on endogenous hormone concentrations or ratios e.g. the T/E ratio are reliable tools to monitor this kind of doping. The results of longitudinal studies of the urinary steroid profile underline that the biosynthesis of the endogenous steroids is tightly controlled and that the metabolism is so constant that the stationary, homeostatic model is appropriate for calculating subject-based reference ranges.

INTRODUCTION

Because the misuse of anabolic androgenic steroids is detectable by screening with GC/MS for the parent compounds or their metabolites [1], endogenous hormones as testosterone or dihydrotestosterone or peptide hormones are used to increase performance.

When searching for endogenous substances in urine as well as in blood we enface first a general problem that the substance(s) administered, the exogenous substance(s), is/are identical or very similar in chemical structure to that produced by the body so that the analytical methods cannot differentiate between applied and endogenous product.

The second problem is a clear differentiation between the concentration found normally in body fluids and the increased level after application of endogneous hormones. The approach used in monitoring the widely used testosterone application is to determine the relation to an endogenous substance not affected by the application i.e. the testosterone/epitestosterone ratio. In both cases, concentrations and ratios, an interpretation is necessary whether the measured value is "normal". The decision limit set by the IOC Medical Commission 1982 for the testosterone/epitestosterone ratio is based on a population-based reference range [2].

USADA 0946

APPROPRIATENESS OF POPULATION-BASED REFERENCE RANGES

The appropriateness of a population-based reference range for the interpretation of an individual measurement can be judged by the ratio of intra- to inter-individual variation of the parameter (ratio r) **[3]**. Steroid profiling from research samples **[4,5]** and out of competition controls of the German decathlon team allow to calculate the ratio r for all parameters of the steroid profile (Table 1).

(1) $$r = \sqrt{\frac{E\sigma^2}{Var\mu_i}}$$

$E\sigma^2$: average of the intra-individual variances ($\approx \overline{s_i^2}$)
$Var\mu_i$: variance of the individual mean values ($= s^2_{\bar{x}_i}$)

Table 1: Relation of the intra- to the interindividual variance (ratio r according to formula 1) of the concentrations [ng/ml] of the endogenous steroids and the concentration ratios (20 members of the German decathlon team, data base 1992) **[6]**
$E\sigma^2$: average of the intra-individual variances
$Var\mu_i$: variance of the individual mean values

	$E\sigma^2$	$Var\mu_i$	Quotient r
AND	0.023	0.018	1.25
ETIO	0.030	0.037	0.79
EPIT	0.028	0.061	0.45
TEST	0.030	0.134	0.22
Adiol	0.033	0.026	1.26
Bdiol	0.073	0.120	0.61
Pregnd	0.032	0.036	0.88
AND/ETIO	0.005	0.036	0.15
TEST/EPIT	0.008	0.179	0.04
AND/TEST	0.010	0.078	0.12
AND/EPIT	0.013	0.146	0.09
ETIO/TEST	0.010	0.132	0.08
ETIO/EPIT	0.010	0.065	0.15
Adiol/Bdiol	0.009	0.076	0.12

USADA 0947

In summary, Harris **[3]** gives the following interpretations for the ratio r:

1. $r \leq 0.6$: the subject based reference range reacts more sensitive to variations of the steroid concentrations and the steroid ratios than the population based reference range.
2. $r > 0.6$ und $r < 1.4$: subject based reference range and population based reference range are in the first approximation equivalent.
3. $r > 1.4$: the population based reference range is more reliable than the subject based reference range, at least for individuals whose variance is equal to the average variance ($E\sigma^2$).

For the steroid concentrations, except for testosterone and epitestosterone, the ratio r lies between 0.6 and 1.4 so that the population based reference range is a useful tool. For the testosterone and epitestosterone concentration and especially for the steroid ratios the ratio r is lower than 0.6 which demonstrates that the population based reference range is quite insensitive for monitoring variations in a person.

In Figure 1 the means of the T/E ratio plus/minus two times the standard deviations of 20 members of the German decathlon team are plotted as well as the population based reference limits (95% range). The means of all athletes are lying within the population based reference range. Only the lower end of two person's distributions are below the lower reference limit. From the Figure 1 it is obvious that the population based reference range is very insensitive to individual variations (compare also the ratio r=0.04). Most of the athletes could have undetected increases of their T/E ratio of more than 4 times their individual standard deviations.

Apart from the variances, the magnitude of the intra-indivudual mean compared to the inter-individual mean is of importance. In doping control, this fact has been considered when monitoring T/E ratios between 6 and 10 which are due to low epitestosterone concentrations. The means of such athletes are lying apart from the population mean at the upper end or outside the distribution of the population. Having only a single measurement there is no way to interpret such a value as physiologically. Only a series of measurements can confirm the measurement as being in agreement with the individual's mean and variance.

In doping control the major advantage of sequential data of athletes's steroid profiles are a more precise interpretation of changes in the steroid ratios than the comparison to population based reference ranges. A prediction of the range in which the next value will fall can be made under the model of homeostasis of the steroid production and excretion. The use of subject

APPROPRIATE STATISTICAL MODEL

In the literature different statistical approaches are described to detect an "outlier" value out of an individual's series of data. Two main models are the homeostasis model and the "random walk" model. The first model presumes a stationary, strictly homeostatic variation so that a current observation be compared with the mean of all previous observations. The nonstationary model uses weighting of the consecutive measurements (time series) so that a current observation be compared with an exponentially smoothed average [7].

The experiences gathered from research projects and from out of competition controls show that the ratios T/E, A/E in individuals are very constant due to the homeostasis of the endocrine steroid biosynthesis [8]. Longitudinal studies on the stability of the steroid profile [4,5] allow a comparison of the variance "between" and "within" a sampling period. 6 male volunteers collected urine samples over night for a period of one month. The first three days within a week were regarded as sampling period so that 4 sampling periods can be compared by ANOVA. In case of a significant F-statistics, a component of variance is attributable to the greater time spans between sampling periods, meaning that a time series model is more appropriate to the problem.

In Table 2 the summarizing descriptive statistics and the results of the analysis of variance for the T/E ratios are given for each volunteer. Figure 2 shows the means and standard deviations of the T/E ratio of 6 male volunteers in each week of the experiment. The means are calculated from the first three days of each week.

USADA 0948

Table 2a: T/E ratio of men, sampling interval 4 weeks. Summarizing statistics and results of analysis of variance (ANOVA) for each of the volunteers 1, 2 and 3. (SS: sum of squares, df: degrees of freedom, MSS: mean sum of squares)

Person 1						
Weeks	*n*	*Sum*	*Mean*	*Variance*	*Stdev*	*Cv%*
1	3	5.38	1.79	0.02	0.15	8.36
2	3	5.98	1.99	0.00	0.05	2.43
3	3	5.31	1.77	0.05	0.22	12.50
4	3	4.89	1.63	0.02	0.14	8.65
ANOVA						
	SS	*df*	*MSS*	*F*	*P*	*critical F*
"between weeks"	0.20	3	0.07	2.88	0.10	4.07
"within weeks"	0.19	8	0.02			
Total	0.39	11				
Person 2						
Weeks	*n*	*Sum*	*Mean*	*Variance*	*Stdev*	*Cv%*
1	3	0.39	0.13	0.0003	0.02	13.86
2	3	0.37	0.12	0.0001	0.01	8.95
3	3	0.40	0.13	0.0004	0.02	15.05
4	3	0.40	0.13	0.0002	0.01	10.34
ANOVA						
	SS	*df*	*MSS*	*F*	*P*	*critical F*
"between weeks"	0.00	3	0.00	0.17	0.92	4.07
"within weeks"	0.00	8	0.00			
Total	0.00	11				
Person 3						
Weeks	*n*	*Sum*	*Mean*	*Variance*	*Stdev*	*Cv%*
1	3	2.95	0.98	0.042	0.20	20.83
2	3	2.73	0.91	0.001	0.03	2.88
3	3	2.58	0.86	0.001	0.03	3.51
4	3	2.86	0.95	0.000	0.02	2.22
ANOVA						
	SS	*df*	*MSS*	*F*	*P*	*critical F*
"between weeks"	0.03	3	0.01	0.77	0.54	4.07
"within weeks"	0.09	8	0.01			
Total	0.11	11				

Table 2b: T/E ratio of men, sampling interval 4 weeks. Summarizing statistics and results of analysis of variance (ANOVA) for each of the volunteers 4, 5 and 6. (SS: sum of squares, df: degrees of freedom, MSS: mean sum of squares)

Person 4						
Weeks	*n*	*Sum*	*Mean*	*Variance*	*Stdev*	*Cv%*
1	3	2.58	0.86	0.00	0.03	3.34
2	3	2.56	0.85	0.00	0.06	7.05
3	3	2.61	0.87	0.01	0.07	8.18
4	3	2.79	0.93	0.03	0.17	18.31
ANOVA						
	SS	*df*	*MSS*	*F*	*P*	*critical F*
"between weeks"	0.01	3	0.00	0.40	0.76	4.07
"within weeks"	0.08	8	0.01			
Total	0.09	11				
Person 5						
Weeks	*n*	*Sum*	*Mean*	*Variance*	*Stdev*	*Cv%*
1	3	3.18	1.06	0.01	0.10	9.16
2	3	3.55	1.18	0.09	0.30	25.66
3	3	2.76	0.92	0.01	0.11	12.10
4	3	2.88	0.96	0.03	0.19	19.38
ANOVA						
	SS	*df*	*MSS*	*F*	*P*	*critical F*
"between weeks"	0.12	3	0.04	1.11	0.40	4.07
"within weeks"	0.30	8	0.04			
Total	0.42	11				
Person 6						
Weeks	*n*	*Sum*	*Mean*	*Variance*	*Stdev*	*Cv%*
1	3	18.08	6.03	0.22	0.47	7.84
2	3	14.35	4.78	0.51	0.71	14.89
3	3	15.62	5.21	0.41	0.64	12.35
4	3	17.16	5.72	0.13	0.36	6.37
ANOVA						
	SS	*df*	*MSS*	*F*	*P*	*critical F*
"between weeks"	2.73	3	0.91	2.85	0.11	4.07
"within weeks"	2.55	8	0.32			
Total	5.28	11				

None of the F-values is significant, i.e. the assumption of the homeostatic model for the T/E ratio is strenthened. One month is a short time period and further investigations are necessary to confirm this result.

USADA 0949

REFERENCES

1. DONIKE, M.; ZIMMERMANN, J.; BÄRWALD, K.-R.; SCHÄNZER, W.; CHRIST, V.; KLOSTERMANN, K.; OPFERMANN, G.: Routinebestimmung von Anabolika in Harn. *Deutsche Zeitschrift für Sportmedizin*, 35 (1984) 14-24.
2. DONIKE, M.; ADAMIETZ, B.; OPFERMANN, G.; SCHÄNZER, W.; ZIMMERMANN, J.; MANDEL, F.: Die Normbereiche für Testosteron- und Epitestosteron-Urinspiegel sowie des Testosteron-/Epitestosteron-Quotienten. In: *Training und Sport zur Prävention und Rehabilitation in der technisierten Umwelt. Eds.: I.-WlFranz, H.Mellerwowicz, W.Noack*, Springer Verlag Berlin (1985) 503.
3. HARRIS, E.K.: Effects of Intra- and Interindividual Variation on the Appropriate Use of Normal Ranges. *Clin.Chem.* 20 (1974), 12, 1535-1542.
4. MARECK-ENGELKE, U.; GEYER, H.; DONIKE, M.: Stability of Steroid Profiles. In: *10th Cologne Workshop on Dope Analysis, 7th to 12th June 1992 - Proceedings, M. Donike, H. Geyer, A. Gotzmann, U.Mareck-Engelke und S. Rauth (Hrsg.)*, Sport und Buch Strauß, Köln (1993), 87-89.
5. MARECK-ENGELKE, U.; GEYER, H.; DONIKE, M.: Stability of Steroid Profiles (2): Excretion Rates From Morning Urines. In: *Recent Advances in Doping Analysis. M. Donike, H. Geyer, A. Gotzmann, U.Mareck-Engelke and S. Rauth (Eds.)*, Sport und Buch Strauß, Köln (1994), 85-89.
6. RAUTH, S.: Referenzbereiche von urinären Steroidkonzentrationen und Steroidquotienten - Ein Beitrag zur Interpretation des Steroidprofils in der Routinedopinganalytik. *Deutsche Sporthochschule Köln:* Dissertation, 1994.
7. HARRIS, E.K.; COOIL, B.K.; SHAKARJI, G.; WILLIAMS, G.Z.: On the Use of Statistical Models of Within-Person Variation in Long-Term Studies of Healthy Individuals. *Clin.Chem.* 26 (1980), 3, 383-391.
8. DONIKE, M., RAUTH, S., MARECK-ENGELKE, U., GEYER, H., AND NITSCHKE, R.: Evaluation of Longitudinal Studies, the Determination of Subject Based Reference Ranges of the Testosterone/Epitestosterone Ratio. In: *Recent Advances in Doping Analysis. M. Donike, H. Geyer, A. Gotzmann, U.Mareck-Engelke and S. Rauth (Eds.)*, Sport und Buch Strauß, Köln (1994), 33-39.
9. HARRIS, E.K.: Some Theory of Reference Values. II. Comparison of Some Statistical Models of Intraindividual Variation in Blood Constituents. In: *Clin.Chem.* 22 (1976), 8, 1343-1350.

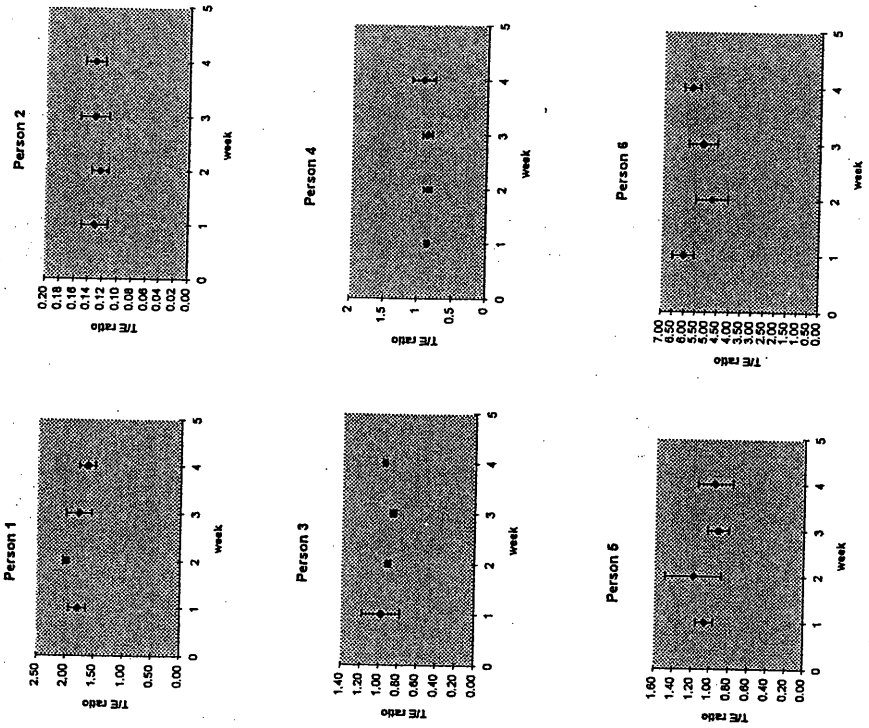


Figure 2: Means and standard deviations of the T/E ratio of each volunteer in each sampling period (week).

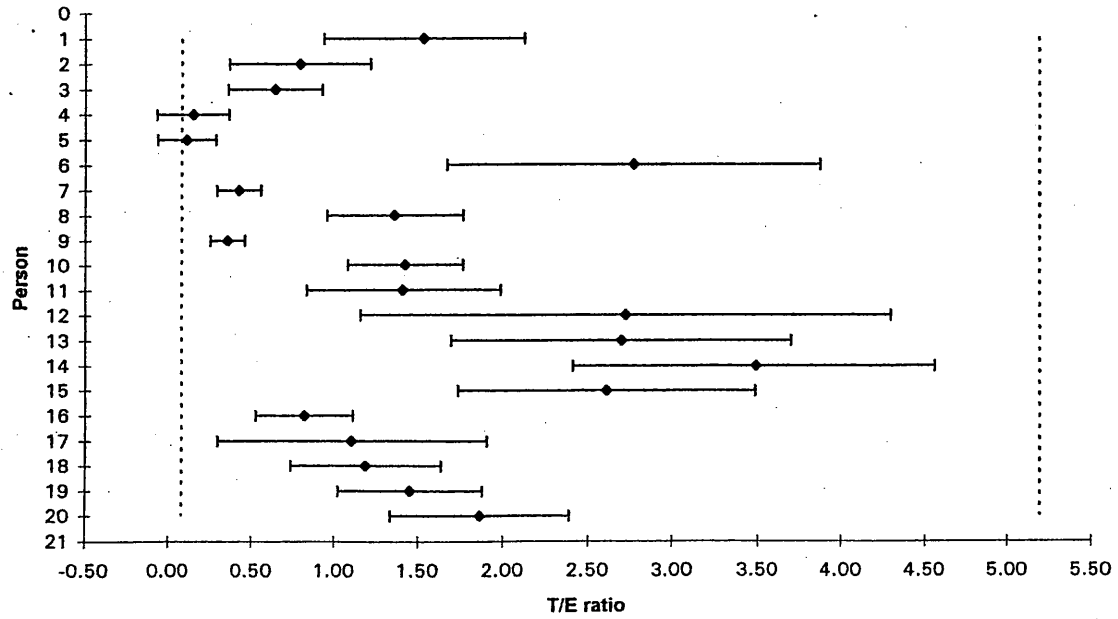


Figure 1: Means (filled diamond) and standard deviations (vertical bars) of the T/E ratio of 20 members of the German decathlon team; lower (0.08) and upper (5.19) reference limits of the population-based reference range [4] are shown as dotted lines.

H. Geyer, W. Schänzer, U. Mareck-Engelke and M. Donike

Factors Influencing the Steroid Profile

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

Abstract

The parameters of the urinary steroidprofile, analysed for dope control purposes, are very stable. These parameters can be influenced by the application of endogenous steroids, anabolic androgenic steroids, probenecid, diuretics, ethanol and trimethoprim. Also bacterial activities in the urine and side activities during the enzymatic hydrolysis may cause changes. All these factors lead to characteristic patterns of the steroidprofile.

Introduction

Steroidprofiling is a well known method of clinical endocrinology, used for detection enzyme deficiencies [1,2]. This method was introduced in the dope analysis by Donike et. al. [3,4] for the detection of the misuse of exogenous testosterone. Several studies have shown, that the steroid profile parameters, especially the steroid ratios used for dope control purposes are very stable. These ratios are not changed by training and severe physical endurance performance [5,6], by circadian rhythm [7], by the menstrual cycle [8] or by circannual rhythm [9]. In the following are presented factors which lead to obvious changes of these parameters and should be taken into account when steroidprofiles are interpreted.

Experimental

Isolation of steroids

2 ml of urine are adsorbed on Amberlite XAD-2 polystyrene resin. The XAD-2 column (pasteur pipette with a glass pearl,XAD-2 bed height: ca. 20 mm) is washed with 2 ml of twice distilled water, and conjugated and unconjugated steroids are eluted with 2 ml methanol. To the methanolic eluate 20 µl of the internal standard solution are added (Tab. 1). The methanolic eluate is evaporated to dryness, and the residue is enzymatically hydrolysed with 50 µl ß-glucuronidase from Escherichia coli K12 (Boehringer, 6800 Mannheim, Germany) in 1 ml 0.2M phosphate buffer, pH 7.0 for 1 hour at 50°C. After hydrolysis, the buffer solution is alkalised with 250 µl 5% potassium carbonate solution, and the steroids are extracted with

USADA 0951

5 ml tert.butylmethyl ether. After centrifugation, the organic layer is transferred into a glass tube and evaporated to dryness under vacuum.

Derivatization for GC/MS

The dry residue is dissolved in 100 µl of MSTFA/NH_4I/ethanethiol,TMS (1000:2:6 v/w/v) and heated for 15 min at 60°C. 3 µl are injected directly into the injection port.

Tab. 1: Internal standard: concentrations of the working solution and resulting concentrations per ml of urine.

reference substances	working solution [µg/ml]	urine [ng/ml]
17α-methyltestosterone	50	500
[2,2,4,4-$^2H_4$]-etiocholanolone	50	500
[16,16,17-$^2H_3$)-testosterone	9	90
[16,16,17-$^2H_3$]-epitestosterone	1.5	15
[2,2,4,4-$^2H_4$]-11ß-hydroxy-androsterone	24	240

GC/MS quantitation

Quantitation of excreted steroids is performed with a GC/MS system [GC/MSD Hewlett-Packard (GC 5890/MS 5971A)], with the electron impact set at 70 eV, column: Hewlett-Packard, HP1, fused silica capillary column cross-linked methyl silicone (OV 1), 17 m, ID 0.20 mm, film thickness 0.11 µm. The carrier gas is helium (1 ml/min, split 1:10), and the temperature program is as follows: initial temperature 180°C, program rate 3°C/min to 229°C, 40°C/min up to final temperature 310°C.

Selected ion monitoring (SIM) is used with the ions presented in Table 2. Urine concentrations are calculated by the peak areas of the detected signals relative to the internal standard [2,2,4,4-$^2H_4$]-etiocholanolone m/z 438. In Figure 1 is presented an printout for a "normal" steroidprofile.

For calibration of the GC/MS instrument, a mixture of the reference substances (Tab. 3) and internal standards (Tab. 1) with the indicated concentrations per ml of urine is derivatized. To avoid wrong calibration factors, caused by a missing bological matrix in the standard we add a mixture of 1-(N,N-diisopropylamine)-alkanes (Dipa) [10]

USADA 0952

Tab. 2: Retention times (RT), retention indices and ion traces of the endogenous steroids which are monitored for steroidprofiling in the Cologne laboratory. Also added are the internal standards (all steroids as per-trimethylsilyl derivatives).

substance	abbreviations	RT	index	m/z
5ß-androstane-3α,17α-diol		8.64	2419	241
5ß-androstane-3ß,17α-diol		8.99	2437	241
5α-androstane-3α,17α-diol		9.12	2433	241
5ß-androstandion		9.19	2437	432
androsterone	AND	10.36	2506	434
d_4-etiocholanolone	d4-ETIO	10.46	2511	438
etiocholanolone	ETIO	10.54	2515	434
5α-androstane-3α,17ß-diol	5αA3αD	10.68	2522	241
5ß-androstane-3α,17ß-diol	5ßA3αD	10.82	2529	241
dehydroepiandrosterone	DHEA	11.66	2572	432
epiandrosterone	EPIAND	11.78	2578	434
5α-androstandion		12.11	2595	432
5-androsten-3ß,17ß-diol		12.13	2596	434
5α-androstane-3ß,17ß-diol	5αA3ßD	12.15	2597	421
d_3-epitestosterone	d3-EPIT	12.15	2597	435
epitestosterone	EPIT	12.19	2599	432
dihydrotestosterone	DHT	12.41	2610	434
4-androstendion		12.85	2633	430
d_3-testosterone	d3-TEST	12.99	2640	435
testosterone	TEST	13.03	2642	432
11ß-hydroxy-androsterone	11OHAN	13.39	2660	522
11ß-hydroxy-etiocholanolone	11OHET	13.65	2673	522
methyltestosterone	MTEST	14.82	2733	446
pregnanediol*		15.37	2761	117
pregnanetriol**		15.98	2792	255

* 5ß-pregnane-3α,20α-diol

** 5ß-pregnane-3α,17α,20α-triol

Tab. 3: Calibration standard: concentrations of the working solution and resulting concentrations per ml of urine.

reference substances	working solution [µg/ml]	urine [ng/ml]
androsterone	200	2000
etiocholanolone	200	2000
testosterone	4	40
epitestosterone	4	40
11ß-hydroxy-androsterone	40	400
11ß-hydroxy-etiocholanolone	20	200
dehydroepiandrosterone	40	400
5α-androstane-3α,17ß-diol	8	80
5ß-androstane-3α,17ß-diol	18	180
pregnandiol*	100	1000
pregnantriol**	100	1000
tetrahydro-cortisol	100	1000
epiandrosterone	10	100
dihydrotestosterone	10	100
Dipa-C_{14} till Dipa-C_{23}	500 each	5000 each

* 5ß-pregnane-3α,20α-diol
** 5ß-pregnane-3α,17α,20α-triol

Results and Discussion

Application of endogenous steroids

After the application of **exogenous TEST** the most obvious changes of the steroidprofile are the increase of the TEST concentration and the ratio TEST/EPIT [3,4] and the decrease of the ratio AND/TEST (Fig. 2). A sample is sucpicious for TEST doping if the steroidprofile values of these parameters exceed the limits of population based reference ranges. Our criteria, based upon reference ranges of 5101 male and 1694 female athletes [11] are presented in Table 4.

Tab. 4: Parameters of the steroidprofile, which make a urine sample suspicious for an application of exogenous TEST.

Parameter	Men	Women
TEST/EPIT	> 6	> 6
AND/TEST	< 20	< 40
c TEST [ng/ml]*	> 130	> 60

*concentration corrected to a specific gravity of 1.020 [11,14]

The application of **exogenous DHT** leads to increases of the excretion of DHT and its 5α-metabolites 5αA3αD, 5αA3ßD, AND and EPIAND [12,13,14]. 5ß-steroids and EPIT are not influenced. Therefore steroidprofiles after the application of DHT are characterized by increased ratios of DHT/ETIO, DHT/EPI, 5αA3αD/5αA3ßD and AND/ETIO (Fig. 3). The criterias for a detection of DHT doping, based on reference ranges of 4631 male and 1341 female athletes [14] are presented in Table 5.

Tab. 5: Parameters of the steroidprofile, which make a urine sample suspicious for an application of exogenous DHT.

Parameter	Men	Women
c DHT [ng/ml]*	>21	> 18
AND/ETIO	> 2.9	< 2.1
5αA3αD/5ßA3αD	> 1.5	> 1.3
DHT/ETIO*	> 8.2	> 8.5
DHT/EPIT	> 0.73	> 2.3

*concentration corrected to a specific gravity of 1.020 g/cm^3 [11,14]
** DHT/ETIO value multiplied with 1000

If **exogenous 5α-androstandion** is administered, the same parameters as after a DHT administeration are changed, but in a different extent (Tab.6). 5α-androstandion itself is not excreted.

USADA 0953

Tab. 6: Maximum values of excretion rates and ratios of a volunteer after administration of 20 mg 5α-androstandion and 25 mg DHT

Parameter	5α-androstandion	DHT
AND [µg/h]	2325	1075
5αA3αD [µg/h]	34.1	61.7
DHT [µg/h]	18.5	65.8
5αA3αD/5ßA3αD	2.8	6.4
AND/ETIO	22.6	8.5

The application of **exogenous AND** increases the excretion of AND and 5αA3αD. No TEST or EPIT is formed. This was shown with an excretion study with [2,2,4,4-$^2H_4$]-AND. Increased ratios AND/ETIO and 5αA3αD/5ßA3αD are the consequence of an AND application.

The use of **exogenous EPIT** increases the excretion of EPIT and of the 17α-diols, 5ß-androstane-3α,17α-diol and 5α-androstane-3α,17α-diol. These results were achieved by excretion studies with EPIT and deuterated EPIT ([2,2,4,6,6-$^2H_5$]-EPIT). Suitable parameters for the detection of an EPIT application maybe the EPIT concentration, the ratios TEST/EPIT and the ratio of the above mentioned 17α-diols to steroids which are not influenced by the EPI-metabolism like the 17ß-diols.

Application of anabolic androgenic steroids (AAS)

The application of AAS reduces the endogenous production androgenic steroids by a negative feedback mechanism leading to a decrease of their urinary excretion [5,15]. This is shown in Figure 4 for three volunteers who self-administered metandienone for 6 weeks in doses from 10mg/day to 40 mg/day, leading to substantial decrease in their urinary excretion rates.

A further effect of a longterm application of AAS is a suppression of the the 5α-reductase activity [5,16]. This effect remains also after cessation of the drug and leads to ratios of AND/ETIO which are far lower than 1 (Fig. 5).

USADA 0954

Application of ethanol

The application of ethanol in high amounts may increase the ratio TEST/EPIT and decrease the ratio AND/TEST by increase of the excretion of TEST glucuronide and decrease of the excretion of AND glucuronide. These changes are connected with the presence of ethanol in urine (Fig. 6). These results have always taken into account if high TEST/EPIT ratios are found in dope control samples, especially in "out of competition" controls. To avoid false positive results, ethanol should be measured in urine, e.g. by Headspace/GC.

Application of probenecid and diuretics

The application of probenecid reduces the excretion of conjugated steroids [5,17]. This is shown in Figure 7 for AND and ETIO. Steroid ratios are not changed. This effect is due to a competitive inhibition of active transport mechanisms of organic acids in the proximal tubuli of the kidneys.

In contrary to probenecid, diuretics don't reduce the steroid excretion. These substances increase the urine flow and therefore also lead to lower steroid concentrations.

Application of trimethoprim- and sulfamethoxazol

Trimethoprim- and sulfamethoxazol-metabolites are eluted in a region, where many endogenous steroids are detected. They may disturb the chromatography of the endogenous steroids and lead to wrong evaluations of the steroidprofile. This is shown in Figure 8 for TEST and EPI. To separate the coeluting substances, we recommend a n-pentane extraction [18]

Bacterial activities in urine samples

The first indications for bacterial activities in urine sample are elevated pH values. The most often observed changes of the steroidprofile in such urines are the coming up of 5α-androstandion and 5ß-androstandion (Fig.9). The formation of these steroids is based on a bacterial deconjugation of AND- and ETIO-glucuronide and a following bacterial 3-hydroxy-steroid-dehydrogenase activity. Because of the bacterial deconjugation, high amounts of steroids, normally excreted as conjugates (as AND and ETIO) can be found in the free form.

Another effect of bacterial activities to the steroidprofile, only rarely observed, is the increase of the TEST concentration leading to elevated TEST/EPIT ratios (Fig. 10). We think, that the formation of TEST results from a bacterial hydrolysis of 5-androsten-3ß,17ß-diol sulphate and a following 3ß-hydroxy-Δ^5 steroid-dehydrogenase and steroid-Δ-isomerase

activity (Fig. 12). The bacterial formed testosterone is not conjugated and can be well separated from the TEST glucuronide by an ether extraction before the hydrolysis.

Incomplete hydrolysis

The time required for complete hydrolysis with ß-glucuronidase from E. coli varies between the different steroid conjugates [5]. TEST and EPI glucuronide are hydrolysed completely within some minutes. AND and ETIO glucuronide are hydrolysed slower, probably depending on the higher concentrations in urine. The hydrolysis of ETIO glucuronide is completed faster than the hydrolysis of AND glucuronide (Fig. 11). After incomplete hydrolysis, these different kinetics lead to characteristic patterns of the steroidprofile. The concentrations of AND and ETIO are low, whereas the concentrations of TEST and EPIT are normal. The corresponding ratios e.g. AND/TEST and AND/EPIT are extremly low. Additionally the ratio AND/ETIO maybe far below 1. Such patterns can be observed after the **application of amineptine**, which leads to an inhibition of the hydrolysis. In the case of amineptine the hydrolysis can be completed by longer incubation time or higher amounts of the enzyme preparation or the use of ß-glucuronidase/arylsulfatase from Helix Pomatia.

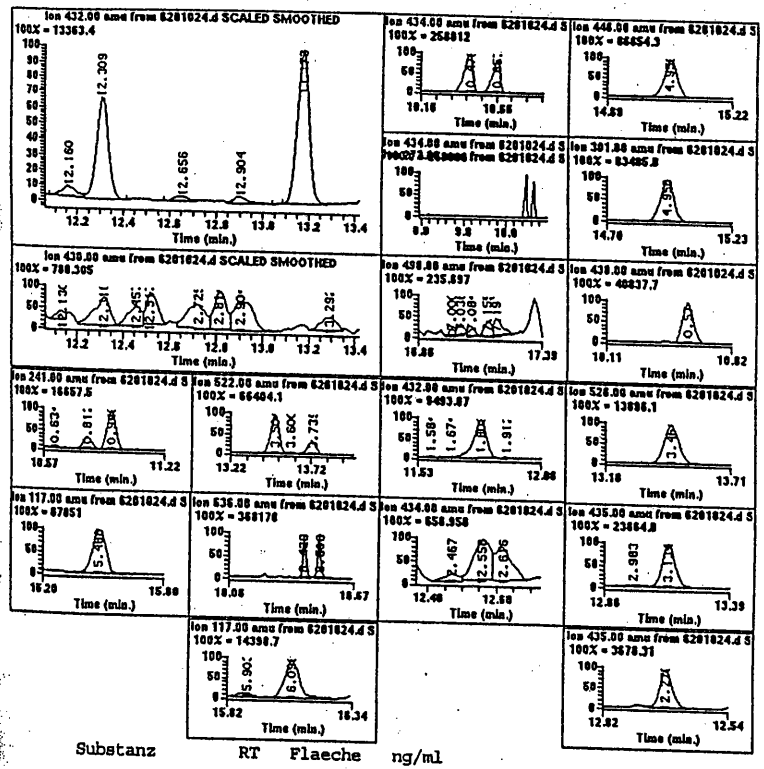
Only about 30 %-50 % of 11OHAN and 11OHET are hydrolysed with ß-glucuronidase from E.coli at 50° C. This is not changed by a prolonged incubation time at this temperature. The hydrolysis at 37°C for 15 hours or the use of ß-glucuronidase/arylsulfatase from Helix Pomatia leads to much higher values [5].

Side activities connected with the enzymatic hydrolysis

The use of ß-glucuronidase/arylsulfatase from Helix Pomatia for the enzymatic hydrolysis may lead to an increased ratios of TEST/EPIT and formation of 4-androstendion [5,19]. Reasons for these changes are side activities (3ß-hydroxy-Δ^5 steroid-dehydrogenase and steroid-Δ-isomerase) in the Helix Pomatia preparation which convert 5-androsten.3ß,17ß-diol to TEST and DHEA to 4-androstendion (Fig.12).

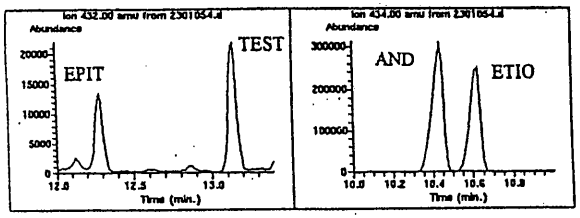
Similar effects can be observed at the hydrolysis with ß-glucuronidase from E.coli. The side activities may stem from bacterias in the phosphate buffer. We could show, that 5-androsten-3ß,17ß-diol, after incubation with an old phospatebuffer was converted to TEST [5]. To avoid suspicious results for TEST/EPIT ratios we recommend to heat the buffer preparation before the use.

USADA 0955

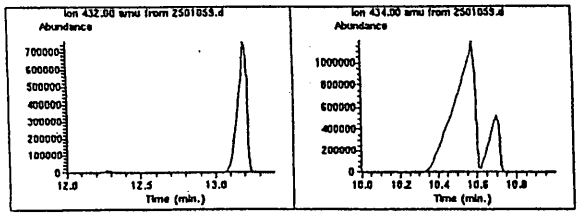


Substanz	RT	Flaeche	ng/ml				
Androsteron	10.48	1043988	2735.1	Methyltest.(446)	14.96	260168	496.5
Etiocholanolon	10.66	762579	1990.1	D4-OH-Androst.	13.46	63027	261.1
Epitestosteron	12.31	32283	34.5	D3-Epitesto.	12.28	12437	14.7
Testosteron	13.17	47353	46.3	D3-Testosteron	13.13	88142	92.5
				D4-Etioch.	10.57	174477	500.0
OH-Androsteron	13.50	239654	1024.3				
OH-Etiochol.	13.74	82277	386.9	Test/Epitest	1.467		
Androstandiol1	10.81	16967	68.5	d3T/d3Epit	7.087		
Androstandiol2	10.94	55035	212.9	Andr./Etio.	1.369		
DHEA	11.80	36741	86.0	Andr./Test.(c)	59.043		
Pregnandiol	15.49	304370	232.9				
TH-Cortisol	18.43	398575	1427.5	D4-Etioch+S	17.08	77	0.04
allo-THC	18.51	391790	1403.2	ISTD 301/446	1.246		
Dihydrotest.	12.47	369	1.9				
Pregnantriol-3	-99.99	-9999	-21.1	Prtriol-3(117)	16.10	61815	1034.6
Pregnantriol-2	-99.99	-9999		Prtriol-2(143)	17.36	2257	

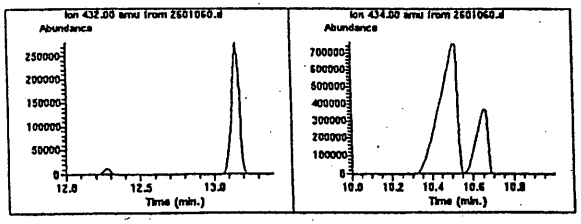
Fig. 1: Screening printout of a "normal steroidprofile" with the ion chromatograms of the endogenous steroids and internal standards.



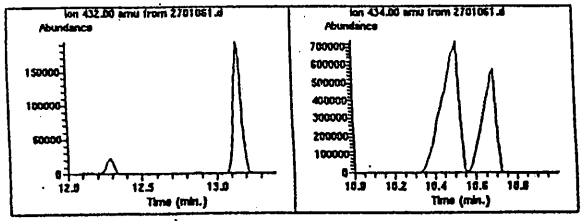
0 h
TEST/EPIT = 1.5
AND/TEST = 42.3
AND/ETIO = 1.3
TEST [ng/ml] = 81



4 h
TEST/EPIT = 65.2
AND/TEST = 7.1
AND/ETIO = 4.6
TEST [ng/ml] = 2916



6 h
TEST/EPIT = 18.8
AND/TEST = 11.8
AND/ETIO = 3.3
TEST [ng/ml] = 964



8 h
TEST/EPIT = 8.0
AND/TEST = 16.2
AND/ETIO = 1.7
TEST [ng/ml] = 664

Fig. 2: Alterations of the steroidprofile after oral application of 40 mg testosterone undecanoate (Andriol R)

USADA 0956

0 h 6 h

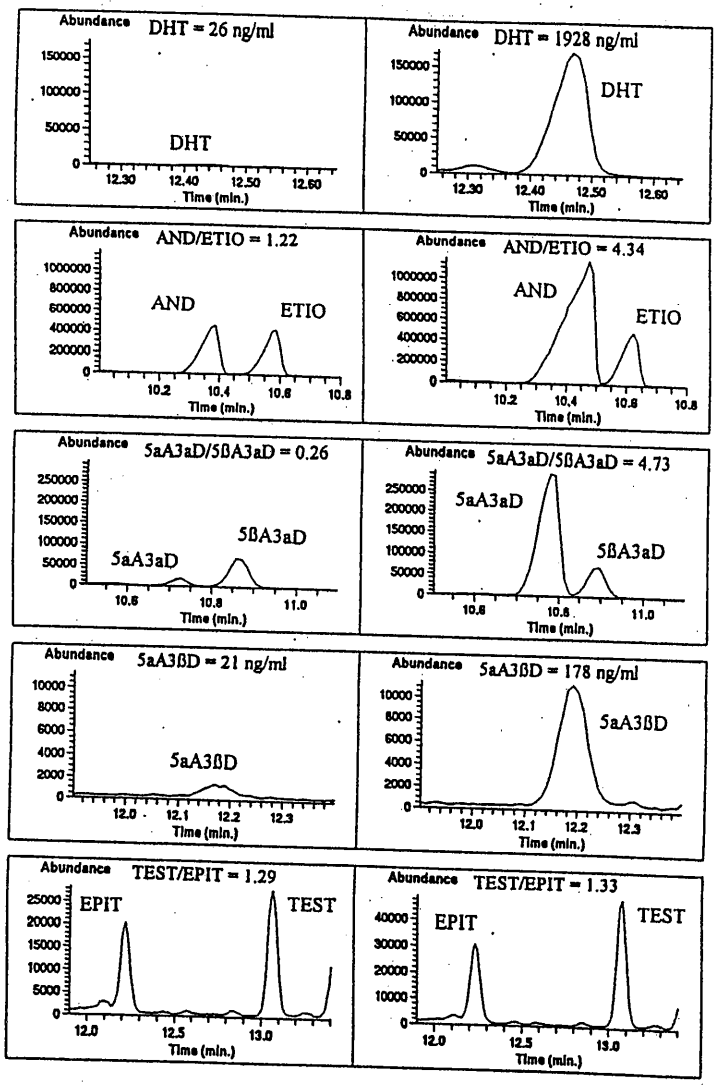


Fig. 3: Alterations of the steroidprofile before and 6 hours after the sublingual application of 25 mg dihydrotestosterone.

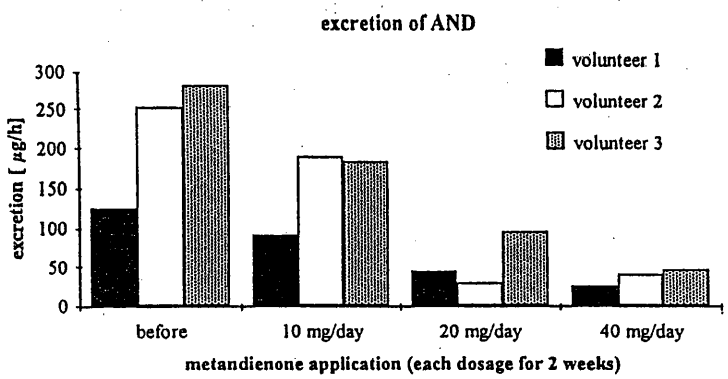


Fig. 4: Decrease of the excretion of androsterone glucuronide after the application of metandienone for 6 weeks with increasing doses.

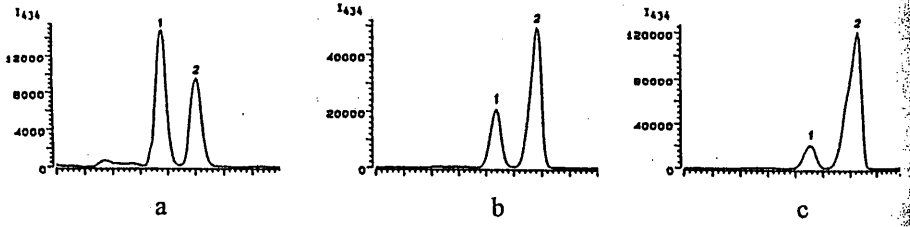
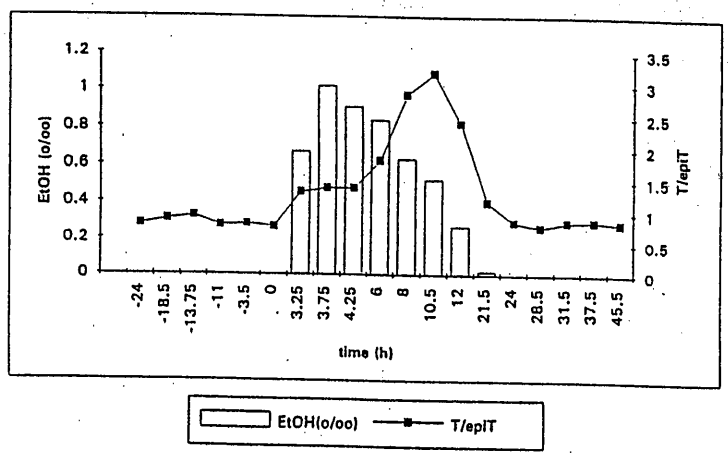


Fig. 5: Change of the ratio androsterone (1)/etiocholanolone (2) after the administration of stanozolol (20 mg/day orally for 8 weeks) was stopped. a) with stanozolol b) 6 days after c) 15 days after

USADA 0957

a)



b)

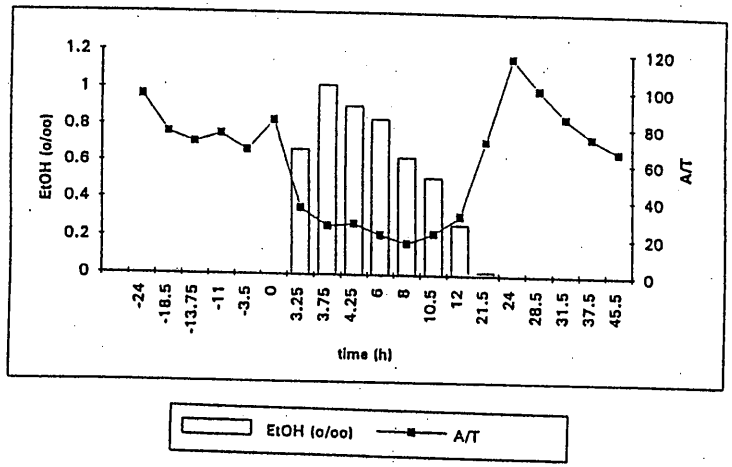


Fig. 6: Changes of the ratios testosterone/epitestosterone (a) and androsterone/ testosterone (b) of a male volunteer after application of 2 g of ethanol per kg bodyweight within 4 hours. Also presented are the found urinary ethanol concentrations (in bars).

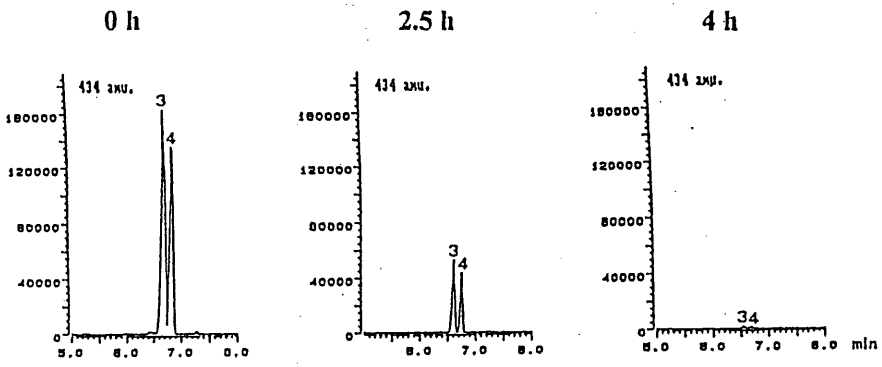


Fig. 7: Decrease of the concentrations of androsterone (3) and etiocholanolone (4) after the application of 3 g of probenecid.

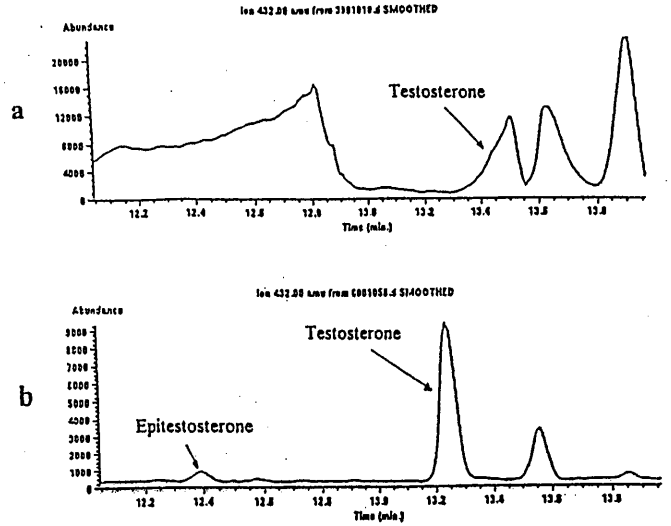


Fig. 8: Single ion chromatogram of ion m/z 432 in the region of testosterone and epitestosterone for an urine containing trimethoprim- and sulfamethoxazol metabolites. a) after ether extraction b) after n-pentane extraction.

USADA 0958

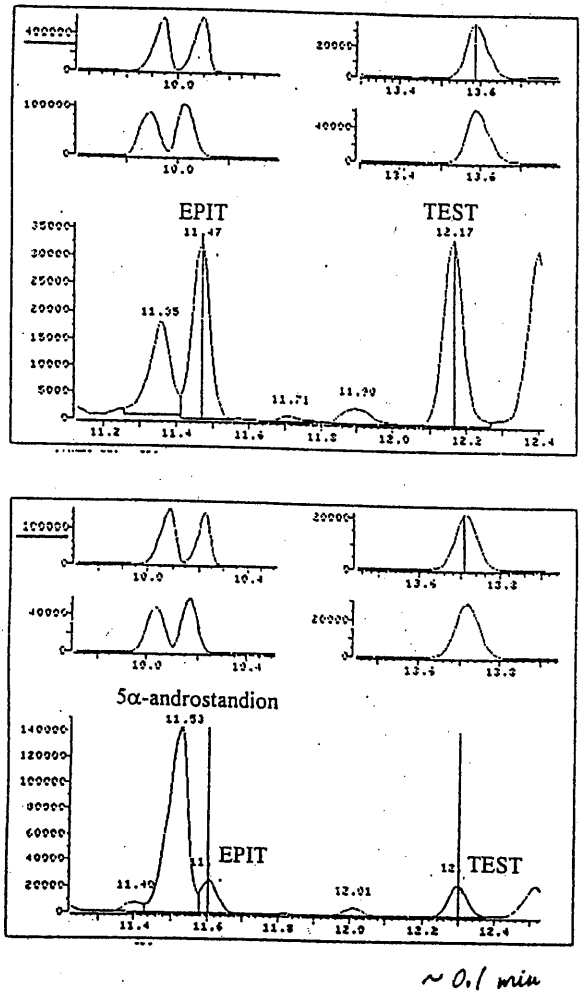
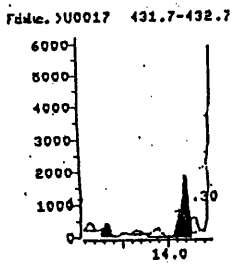
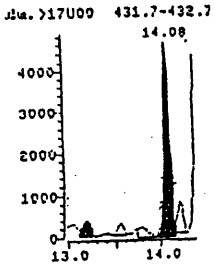


Fig. 9: Changes of the steroidprofile by bacterial activities. 10 ml of a quality control urine were spiked with 0.5 ml bacteria urine. After 48 hours at 30° C high amounts of 5α-androstandion were found.



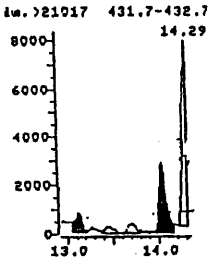
pH 5

combined fraction
Q T/E = 5.3

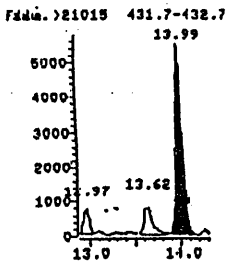


storage 6 months + 4°C
pH 8.9

combined fraction
Q T/E = 9.8

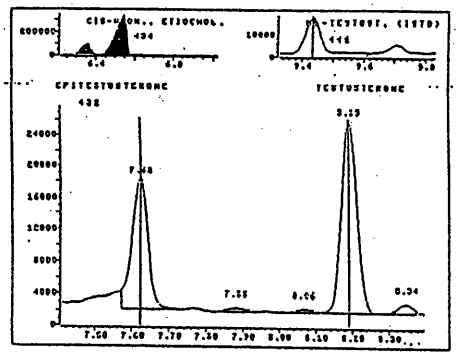


conjugated fraction
Q T/E = 4.9



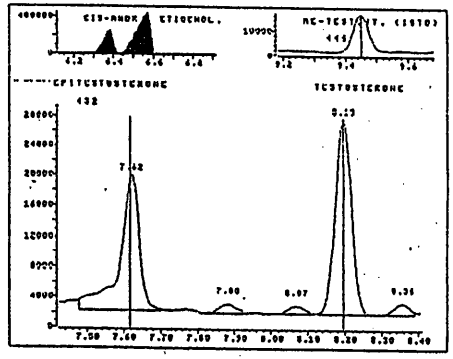
free fraction
with testosterone

Fig. 10: Formation of testosterone and increase of the ratio testosterone/epitestosterone (Q T/E) by bacterial activities in a female urine. High amounts of testosterone were found in the free fraction.



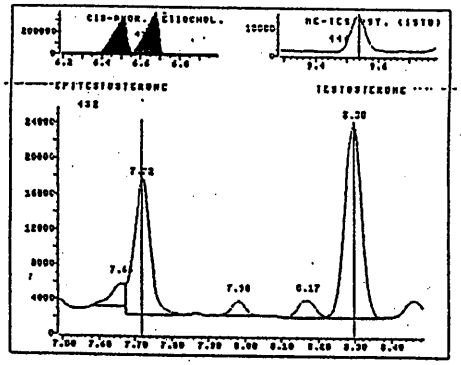
1 min 50°C

Q And/Etio = 0.21



3 min 50°C

Q And/Etio = 0.53



20 min 50°C

Q And/Etio = 1.00

Fig. 11: The dependence of the ratio androsterone/etiocholanolone (Q And/Etio) from the duration of the hydrolysis with ß-glucuronidase from E. coli at 50° C.

USADA 0959

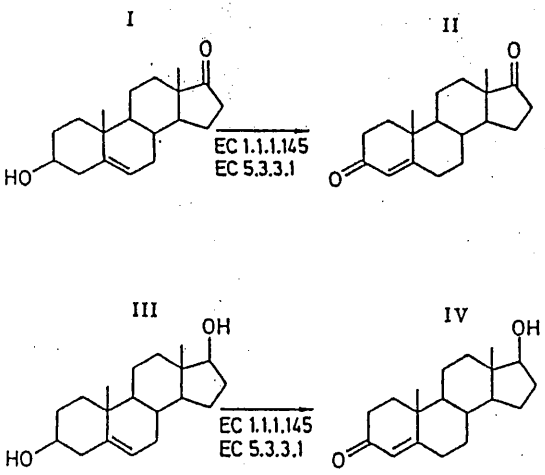


Fig. 12: Conversion of dehydroepiandrosterone (I) to 4-androstendion (II) and 5-androsten-3ß,17ß-diol (III) to testosterone (IV) by activities of a 3ß-hydroxy-Δ^5-steroid-dehydrogenase and steroid-Δ-isomerase.

References

1. Shackleton, C.H.L.: Profiling steroid hormones and urinary steroids - review. J. Chromat. 379 (1986) 91-156.
2. Vestergaard, P.; Sayegh, J.F.; Mowat, J.H.; Hemmingsen, L.: Estimation after multi-column liquid chromatography of common urinary neutral steroids with an application to the assay of plasma 17-oxosteroids. Acta Endocrin. 88 (1978)
3. Donike, M.; Bärwald, K.-R.; K.-R.; Klostermann, K.; Schänzer, W; Zimmermann, J.: Nachweis von exogenem Testosteron. in: Sport: Leistung und Gesundheit. Edt.: H.Heck, W.Hollmann, H.Liesen, R.Rost, Deutscher Ärzte Verlag Köln (1983) 293.
4. Zimmermann, J.: Untersuchungen zum Nachweis von exogenen Gaben von Testosteron Hartung-Gorre Verlag Konstanz, Dissertation Deutsche Sporthochschule Köln, 1986
5. Geyer, H.: Die gas-chromatographisch/massenspektrometrische Bestimmung von Steroidprofilen im Urin von Athleten. Dissertation, Deutsche Sporthochschule Köln, 1990
6. Nitschke, R.: Steroidprofile und Veränderungen biochemischer Parameter bei Hochleistungsradrennfahrern während zwei Rundfahrten. Dissertation, Deutsche Sporthochschule Köln, 1996.
7. Mareck-Engelke U., Geyer H. and Donike M.: Stability of steroid profiles (3) :The Circadian Rhythm of Urinary Ratios and Excretion Rates of Endogenous Steroids in Male. in: M.Donike, H.Geyer, A.Gotzmann, U.Mareck-Engelke (Eds.) Recent advances in doping analysis (2) Sport und Buch Strauß, Köln (1995).

USADA 0960

8. Mareck-Engelke U., Geyer H. and Donike M.: Stability of steroid profiles (4) :The Circadian Rhythm of Urinary Ratios and Excretion Rates of Endogenous Steroids in Female and its Menstrual Dependency. in: M.Donike, H.Geyer, A.Gotzmann, U.Mareck-Engelke (Eds.) Recent advances in doping analysis (2) Sport und Buch Strauß, Köln (1995).
9. Mareck-Engelke U., Flenker U. and Donike M.: Stability of steroid profiles (5) : The Annual Rhythm of Urinary Ratios and Excretion Rates of Endogenous Steroids in Female and its Menstrual Dependency. in: M.Donike, H.Geyer, A.Gotzmann, U.Mareck-Engelke (Eds.) Recent advances in doping analysis (3) Sport und Buch Strauß, Köln (1996)
10. Geyer, H., Mareck-Engelke, U., Nolteernsting, E., Opfermann, G., Donike, M.: The matrix problem in calibration of the GC/MS for endogenous steroids. in: Donike, M., Geyer, H., Gotzmann, A., Mareck-Engelke, U. (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln 1995.
11. Rauth, S.: Referenzbereiche von urinären Steroidkonzentrationen und Steroidquotienten - Ein Beitrag zur Interpretation des Steroidprofils in der Routinedopinganalytik. Dissertation, Deutsche Sporthochschule Köln 1994.
12. Southan, G.J.; Brooks, R.V.; Cowan, D.A.; Kicman, A.T.; Unnadkat, N.; Walker, C.J.: Possible indices for the detection of the administration of dihydro-testosterone to athletes. J. Steroid Biochem. Molec. Biol. 42 (1992) 87-94.
13. Geyer, H.., Schänzer, W., Schindler, U.: Changes of the urinary steroidprofile after sublingual application of dihydrotestosterone. in: Donike, M., Geyer, H., Gotzmann, A., Mareck-Engelke, U. (eds.) Recent advances in doping analysis (3). Sport und Buch Strauß, Köln 1996.
14. Donike, M., Ueki, M., Kuroda, Y., Geyer, H., Nolteernsing, E., Rauth, S., Schänzer, W., Schindler, U., Völker, E., Fujisaki, M.: Detection of dihydrotestosterone (DHT) doping - Alterations in the steroid profile and reference ranges for DHT and its 5α-metabolites. J. Sports.Med.Phys.Fitness, in press.
15. Donike, M.; Geyer, G.; Schänzer, W.; Zimmermann, J.: Die Suppression der endogenen Androgenproduktion durch Metandienon. in: I.-W.Franz, H.Mellerowicz, W.Noack (Eds.). Training und Sport zur Prävention und Rehabilitation in der technisierten Umwelt Springer Verlag Berlin Heidelberg 1985
16. Donike, M.; Geyer, G.; Kraft, M.; Rauth, S.: Longterm Influence of Anabolic Steroid Misuse on the Steroid Profile. in: P.Belotti, G.Benzi, A.Ljungqvist (eds.). Official proceedings. IInd International Athletic Foundation World Symposium on Doping in Sport, Monte Carlo 1989.
17. Geyer, H., Schänzer, W., Donike, M.:Probenecid as masking agent in dope control-Inhibition of the urinary excretion of steroid glucuronides.in: Donike, M., Geyer, H., Gotzmann, A., Mareck-Engelke, U., Rauth, S. (eds.) 10th Cologne workshop on dope analysis 7th to 12th june 1992. Sport und Buch Strauß, Köln 1993.
18. Geyer, H., Mareck-Engelke, U., Schänzer, W., Donike, M.: Simple purification of urine samples for improved detection of anabolic and endogenous steroids. in: Donike, M., Geyer, H., Gotzmann, A., Mareck-Engelke, U. (eds.) Recent advances in doping analysis. Sport und Buch Strauß, Köln 1994.
19. Kuoppasalmi, K., Leinonen, A., Kajalainen, U.: Detection of exogenous testosterone in doping analysis:methodological aspects. in: Sports medicine and exercise science. Proceedings of the Olympic scientific congress. Eugene, Oregon, 1984.

H. Geyer, U. Mareck-Engelke, W. Schänzer and M. Donike

The Cologne protocol to follow up high testosterone/ epitestosterone ratios

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

This lecture was held at the 11th and 12th Cologne Workshop on Dope Analysis 1993 and 1994.

Introduction

The most important parameter for the detection of doping with exogenous testosterone is an elevated ratio testosterone/epitestosterone (1, 2). Before a dope control sample is declared positive for testosterone, it has to be proven, according to the rules of the IOC (3), that the elevated ratio testosterone/epitestosterone is not natural. Therefore we have developped in cooperation with the Antidoping Commission of the International Cycling Federation (UCI) a complex protocol to follow up high testosterone/epitestosterone values. The main intention of this protocol is the prevention of false positive results.

The knowledge of the „normal" steroidprofile as basis for the detection of doping with testosterone

The basis for the detection of doping with testosterone and other endogenous steroids is the knowledge of the „normal" steroid profile, i.e. population based reference ranges of endogenous steroids. Therefore we have analysed within our screening procedure for anabolic steroids steroidprofiles of dope control urines for several years. All samples were analysed and quantified with the same method, which was described in detail several times (4, 5). In table 1 are presented the endogenous steroids, which are monitored for steroidprofiling in the Cologne laboratory.

USADA 0961

Tab. 1: Retention times, retention indices and ion traces of the endogenous steroids which are monitored for steroidprofiling in the Cologne laboratory. Also added are the internal standards (all steroids as per-trimethylsilyl derivatives).

Substanz	Abbreviations	RT	Index	m/e
5ß-androstane-3α,17α-diol		8.64	2419	241
5ß-androstane-3ß,17α-diol		8.99	2437	241
5α-androstane-3α,17α-diol		9.12	2433	241
5ß-androstandion		9.19	2437	432
d_5-androsterone	d5-AND	10.28	2501	439
androsterone	AND	10.36	2506	434
d_4-etiocholanolone	d4-ETIO	10.46	2511	438
etiocholanolone	ETIO	10.54	2515	434
5α -androstane-3α,17ß-diol	5αA3αD	10.68	2522	241
5ß-androstane-3α,17ß-diol	5ßA3αD	10.82	2529	241
dehydroepiandrosterone	DHEA	11.66	2572	432
epiandrosterone	EPIAND	11.78	2578	434
5α-androstandion		12.11	2595	432
5-androsten-3ß,17ß-diol		12.13	2596	434
5α -androstane-3ß,17ß-diol	5αA3ßD	12.15	2597	421
d_3-epitestosterone	d3-EPIT	12.15	2597	435
epitestosterone	EPIT	12.19	2599	432
dihydrotestosterone	DHT	12.41	2610	434
4-androstendion		12.85	2633	430
d_3-testosterone	d3-TEST	12.99	2640	435
testosterone	TEST	13.03	2642	432
11ß-hydroxy-androsterone	11OHAN	13.39	2660	522
11ß-hydroxy-etiocholanolone	11OHET	13.65	2673	522
methyltestosterone	MTEST	14.82	2733	446
pregnanediol*		15.37	2761	117
pregnanetriol**		15.98	2792	255

* 5ß-pregnane-3α,20α-diol

** 5ß-pregnane-3α,17α,20α-triol

The descriptive statistics and the calculated population based reference ranges of the steroidprofile parameters are presented in table 2-5. All values and details of the statistical calculation (reference population, reference sample group etc.) are published in the thesis of S. RAUTH (6). The calculation of the population based reference ranges were performed according to a concept and to recommendations of the International Federation of Clinical Chemistry (IFCC). All concentrations are corrected to a urine density of 1.020 g/cm^3.

USADA 0962

Tab. 2: Descriptive statistics of the concentration of endogenous steroids and the concentration of the reference sample group men. (6)

concentrations	N	$\tilde{x}$ [ng/ml]	10.Perc. [ng/ml]	90.Perc. [ng/ml]	$\bar{x}$ [ng/ml]	s [ng/ml]	skewness	excess
AND	5101	2671	1322	4986	2975	1545.23	1.31	2.94
ETIO	5101	2032	1020	3797	2279	1209.92	1.58	4.88
EPIT	5095	27.7	9.2	70.6	35.7	30.03	2.77	15.49
TEST	5100	36.8	6.3	88.3	44.6	37.94	2.78	20.06
Adiol	5088	49.6	22.1	108.3	60.4	44.87	3.84	33.30
Bdiol	5088	128.1	38.0	342.8	166.5	141.79	2.28	9.26
Pregnd	5088	279.9	118.0	616.7	335.0	231.51	2.39	11.82
ratios	N	$\tilde{x}$	10.Perc.	90.Perc.	$\bar{x}$	s	skewness	excess
AND/ETIO	5283	1.32	0.77	2.22	1.43	0.60	1.21	2.78
TEST/EPIT	5277	1.35	0.25	3.29	1.65	1.41	3.54	41.49
AND/TEST	5282	72.5	32.7	400.5	191.0	620.82	23.46	904.87
AND/EPIT	5283	94.0	43.0	244.6	126.7	110.18	4.03	34.93
ETIO/TEST	5283	54.9	24.8	294.8	143.2	480.82	26.94	1120.79
ETIO/EPIT	5277	72.9	30.1	195.3	99.9	99.06	5.54	67.61
Adiol/Bdiol	5283	0.41	0.17	1.03	0.52	0.41	3.60	41.22

Tab. 3: Descriptive statistics of the concentrations of the endogenous steroids and the concentration ratios of the reference sample group women. (6)

concentrations	N	$\tilde{x}$ [ng/ml]	10.Perc. [ng/ml]	90.Perc. [ng/ml]	$\bar{x}$ [ng/ml]	s [ng/ml]	skewness	excess
AND	1696	1859	802	4199	2286	1629.40	2.22	7.99
ETIO	1696	1990	891	4050	2326	1548.73	3.15	27.53
EPIT	1693	6.0	2.1	22.1	10.0	12.72	4.97	41.95
TEST	1693	8.2	2.1	25.5	12.6	15.21	4.09	24.86
Adiol	1694	18.5	8.2	48.1	25.1	24.30	3.76	20.77
Bdiol	1694	64.1	18.1	190.3	92.5	92.88	2.70	10.90
Pregnd	1695	390.5	146.2	1320.6	642.5	884.49	4.98	36.29
ratios	N	$\tilde{x}$	10.Perc.	90.Perc.	$\bar{x}$	s	skewness	excess
AND/ETIO	1742	0.96	0.58	1.59	1.04	0.44	1.47	4.53
TEST/EPIT	1736	1.34	0.34	3.87	1.83	1.82	3.90	31.34
AND/TEST	1739	220.6	82.2	777.0	449.9	1384.28	21.15	621.99
AND/EPIT	1742	304.3	119.0	657.7	355.4	230.63	1.45	3.31
ETIO/TEST	1742	232.1	90.0	746.3	462.7	1181.14	12.35	207.04
ETIO/EPIT	1739	321.5	109.0	812.9	399.5	301.30	1.56	3.06
Adiol/Bdiol	1742	0.31	0.11	0.84	0.41	0.36	4.80	61.35

Tab. 4: Reference limits of the non-parametric 95% reference range and the respective 90% confidence intervals for the reference sample group of men. ($N_{(concentrations)}$=5101, $N_{(ratios)}$=5283 (6)

	Reference limits		90% confidence interval			
	2.5%	97.5%	lower limit		upper limit	
concentrations	[ng/ml]	[ng/ml]	[ng/ml]		[ng/ml]	
AND	867.2	6703	834.5	894.3	6533	6881
ETIO	673.7	5294	654.3	697.1	5107	5497
EPIT	4.9	112	4.56	5.39	106.7	118.4
TEST	2.06	137.4	1.87	2.18	131.4	143.8
5αA3αD	13.85	166.5	13.15	14.72	161.4	173.3
5ßA3αD	19.63	550.1	18.44	21.29	531.3	572.8
Pregnd	73.2	951.2	70.6	77.2	910.7	984.1
	Reference limits		90% confidence interval			
ratios	2.5%	97.5%	lower limit		upper limit	
AND/ETIO	0.55	2.869	0.53	0.57	2.81	2.93
TEST/EPIT	0.08	5.19	0.07	0.08	5.01	5.4
AND/TEST	22.5	1164	21.7	23.2	1052	1269
AND/EPIT	27.7	406.3	26.6	28.7	386.8	426.7
ETIO/TEST	16.7	819.2	16.3	17.2	768.6	905.9
ETIO/EPIT	20.5	345.8	19.92	21.42	326.8	362.3
Adiol/Bdiol	0.11	1.56	0.11	0.12	1.51	1.61

USADA 0963

Tab. 5: Reference limits of the non-parametric 95% reference range and the respective 90% confidence intervals for the reference sample group of women. ($N_{(concentrations)}$=1694, $N_{(ratios)}$=1742) (6)

	Reference limits		90% confidence interval			
	2.5%	97.5%	lower limit		upper limit	
concentrations	[ng/ml]	[ng/ml]	[ng/ml]		[ng/ml]	
AND	404.1	6439	368.8	458.4	6135	6964
ETIO	473.2	6107	395.2	503.1	5698	6564
EPIT	1.11	42.2	1.04	1.21	37.2	47.9
TEST	0.65	57.3	0.55	0.83	48.1	62.1
5αA3αD	4.74	91	4.12	5.14	83.1	98.3
5ßA3αD	9.14	366.4	7.97	10.1	312.4	387.8
Pregnd	83.1	3089	76.9	89.3	2659	3382
	Reference limits		90% confidence interval			
ratios	2.5%	97.5%	lower limit		upper limit	
AND/ETIO	0.42	2.15	0.39	0.44	2.03	2.22
TEST/EPIT	0.11	6.31	0.1	0.13	5.91	6.7
AND/TEST	47.9	2184	40.0	52.1	1704	2591
AND/EPIT	65.3	939.4	60	74.3	893.9	986.8
ETIO/TEST	37.0	2397	31.8	42.0	1998	2905
ETIO/EPIT	60.5	1205	53.1	65.0	1152	1289
Adiol/Bdiol	0.07	1.24	0.07	0.08	1.16	1.28

Parameters for the detection of doping with testosterone

The application of exogenous testosterone leads to characteristic changes of the normal steroidprofile (1). The most obvious change is the **increase of the ratio TEST/EPIT**. A value of this parameter above the upper limit of the reference range, i.e. higher than 6, indicates a suspicion for testosterone doping. Beside the TEST/EPIT ratio, two other parameters are important for the detection of testosterone doping. These parameters are a **decreased ratio of AND/TEST** and an **increased concentration of TEST (cTEST).**

If all three parameters are outside of the limits of the reference values (see table 6) and other influences, e.g. bacterial activities, can be excluded, there is a high probability, that the unnormal steroidprofile is caused by a testosterone application. But because the T/E ratio is the most sensitive parameter for the detection of doping with testosterone, a suspicion is already given, if the T/E ratio is higher than the upper reference limit.

Tab. 6: Parameters of the steroidprofile, which make a urine suspicious for an application of exogenous TEST.

Parameter	Men	Women
TEST/EPIT	> 6	> 6
AND/TEST	< 20	< 40
c TEST [ng/ml] *	> 130	> 60

* concentration corrected to a urine density of 1.020

To study, if the high T/E ratio is natural, caused by a testosterone application or caused by other influences, e.g bacterial, pathological or analytical influences, we start with the following protocol.

Screening results -Search for other factors which may increase the ratio TEST/EPIT

If there was found a ratio TEST/EPIT > 6 we look first in our screening results for other factors, which may lead, beside the TEST application, to increased TEST/EPIT ratios or change the other testosterone related parameters AND/TEST and cTEST. In our screening method we have included some controls to recognize these factors:

Mistakes in the sample preparation and changes of the GC/MS conditions may change the TEST/EPIT ratio and the other testosterone related parameters. This is controled by the use of the internal standards d3-TEST, d3-EPIT and d4-ETIO. The d3-TEST and d3-EPIT are

USADA 0964

added to each sample in a ratio of 6:1 (90 ng/ml / 15 ng/ml) so that the ratios TEST/EPIT can be corrected (7).

Bacterial activities in the urine can be recognized by the presence of 5α- and 5ß-androstandion (4), which are monitored in our screening procedure. Also high amounts of DHT are found. The AND/TEST ratios are often low. These changes of the steroidprofile are mostly connected with high pH values. Further studies of such samples have shown, that in all of these samples high amounts of the steroid glucuronides are hydrolysed, and that the urine contains different enzyme activities as 3-hydroxy-steroid-dehydrogenase activity or steroid-Δ-isomerase etc. which can transform unconjugated androsterone to 5α-androstandion or unconjugated 5-androstendiol to testosterone (5). In these cases, the steroidprofile parameters are worthless and we stop the follow up protocol. We inform the federation about the presence of bacterias, which are mostly caused by insuitable transport or storage conditions and recommend to repeat the dope controls and to improve the transport and storage conditions.

Bacterial activities in the phosphate buffer used for the hydrolysis can not be recognized from our screening chromatograms. We control such side activities in the hydrolysis step by spiking a buffer solution with 5 µg of 5-androstene-3ß,17ß-diol and DHEA (8). If TEST or 5-androstendion is found after incubation of 1 hour at 50° C, the sample preparation has to be repeated.
We prevent such side activities by heating the buffer for several minutes before use (8).

Incomplete hydrolysis leads to a low and therefore suspicious ratio AND/TEST because AND-glucuronide is hydrolysed slower than TEST-glucuronide (5). We control the hydrolysis with the internal standard [2,2,3,4,4-$^{2}H_{5}$]-androsterone-D-17-glucuronide, which is added in an amount of 500 ng/ml to each sample. In case of an incomplete hydrolysis, we repeat the sample preparation.

Incomplete derivatisation may lead to wrong steroidprofile values, e.g. to low AND/TEST values. The derivatisation is controled by the monitoring of the ion 272 (molecular ion -90) for AND and ETIO, mono-TMS. If these two substances are detected, the derivatisation is

incomplete and we add to the derivatised sample (ca. 80 µl) once more 20 µl of derivatisation reagent with a higher concentration of the catalast NH_4I (2%).

If we have excluded all above mentioned factors for the high TEST/EPIT ratio we start with the confirmation of the sample.

Confirmation of suspicous TEST/EPIT samples

Our procedure for the confirmation of samples with high TEST/EPIT ratios was exactly described by NOLTEERNSTING et al. (7).

The most important points are:

Reproducibility

To examine the reproducibility of the method, we prepare three aliquots of the sample which are injected twice. The variation coefficient of the TEST/EPIT ratios should be better than 5%. The mean of the TEST/EPIT values of the replicates minus tthree times the standard deviation should result in a value higher than 6.

Correction with deuterated standards

For the correction of the ratios TEST/EPIT the internal standards d3-TEST/d3-EPIT 6:1 are added to each sample.

Full scan spectrum of TEST and EPIT

Additional to the 3 aliquots we prepare one aliquot without internal standard to achieve full scan spectras of TEST and EPIT. This is done to show, that no other substance is coeluted with TEST or EPIT.

Increased urine volume

The used urine volume is increased to achieve a good GC signal especially for EPIT.

USADA 0965

Separation of the free fraction

Before the hydrolysis, the free steroids are separated by extraction with ether. By this step, we prevent false positive results by unconjugated, bacterial produced, TEST (5).

Heating of the buffer

To avoid testosterone producing side activities from the phosphate buffer (8) during the hydrolysis, we boil the phosphate buffer for several minutes before the use.

Hydrolysis with ß-glucuronidase from E.coli

For the hydrolysis of the glucuronides we only use ß-glucuronidase from E. coli, because we have never observed testosterone producing side activities in this enzyme preparation. Such activities can be found in the ß-glucuronidase/ aylsulfatase of Helix Pomatia preparations (9).

n-pentane extraction

After hydrolysis the extraction of the steroids is performed with n-pentane instead of tert. butylmethyl ether. This is an additional clean up step to reduce the biological background for the detection of TEST and EPIT (10).

Changed MS parameters

For the MS detection is used a SIM method with only 5 ions. The molecular ions of the bis-TMS derivatives of TEST, EPIT, d3TEST and d3EPIT are registered with dwell times of 200 msec (7).

Measurement of LH, FSH and HCG

As proposed by KICMAN et al.(11) and COWAN et al.(12) we analyse also the gonadotropines LH, FSH and HCG. Of most importance is the ratio LH/TEST.

Measurement of e.. .ol

Several studies have shown that the application of ethanol may increase the TEST/EPIT ratio and decrease the AND/TEST ratio (13). Additionally we have found, that these changes of the steroidprofile are always connected with the presence of ethanol in urine (13). Therefore we analyse samples with elevated TEST/EPIT ratios also for ethanol by Headspace/GC.

If we find ethanol, we stop the follow up protocol and don't give the sample positive. Until now we observed such cases only in Out of Competition Controls.

If the TEST/EPIT ratio > 6 is confirmed and the additional analyses are performed, we continue with the following steps.

Information of the Federation and request for further data

We inform the federation about the high TEST/EPIT ratio and ask for retrospective (code numbers of former dope controls) or prospective data (further dope controls) . For retrospective data, which are archived in other laboratories, we recommend the federation to provide the information listed in the formsheet for high TEST/EPIT values (Fig. 1).
For the decision making process, we need at least data from 4 samples of the same athlete.
The statistical evaluation of the datas is performed as presented in the following chapter

If the retrospective or prospective data are not sufficient for the decision (e.g. not enough data, strong variation of the data), an endocrinological study is performed.

USADA 0966

Elevated TEST/EPIT value

hg/doku/s41/inafte

Laboratory	
Sample Code	
Laboratory Code	
Event	
Date of dope control	
Declaration of medication	

Screening Results

pH	
specific gravity [g/cm^3]	
androsterone [ng/ml]	
etiocholanolone [ng/ml]	
testosterone [ng/ml]	
epitestosterone [ng/ml]	
5α-androstane-3α,17ß-diol [ng/ml]	
5ß-androstane-3α,17ß-diol [ng/ml]	
LH [miU/ml]	
ratio of testosterone / epitestosterone (corrected)	
ratio androsterone / testosterone	
ratio androsterone / etiocholanolone	
ratio testosterone / LH [nmol/lU]	

Screening method

fraction (free/conjugated/combined)	
enzyme preparation for hydrolysis	
correction of ratio testosterone/epitestosterone (deuterated standards, calibration curve)	

Confirmation results

ratio testosterone/epitestosterone	

Confirmation method

fraction (free/conjugated/combined)	
enzyme preparation for hydrolysis	
extraction (n-pentane/ether)	
correction of ratio testosterone/epitestosterone (deuterated standards, calibration curve)	
mass spectrum of testosterone, epitestosterone	

Remarks

Fig. 1: Formsheet for collection of datas from other laboratories.

ndocrinological stud, (ES)

he aim of an ES is to calculate the subject based refernce range for the TEST/EPIT ratio and ıe normal variation of the other TEST related steroid ratios. The basis for the judgement of ıe results is our knowledge about the stability of steroidprofile parameters in rest and xercice (14, 15, 16). The decision, positive or negative for the suspicious sample, is based pon a comparison of the steroidprofile values of the suspicious sample and the values of the S.

he ES takes 2 days .During this time the athlete lives near by the laboratory. He can keep ormal nutrition and training. The application of ethanol is not permitted.

rocedure

Information of athlete about the aim and the procedure of the ES.
Answering of a questionnaire (see Fig. 2)
Collection of all urine fractions in a time period of at least 48 hours (ca. 13 - 15 samples according to a collection schedule (see Fig. 3).
At least 5 urine collections have to take place under observation of a member of the laboratory staff.
Single blood sample collection (10 ml) performed by medical staff.

nalysis

arameter	*Single parameter*	*Analysentechnik*
Steroidprofile parameters in urine concentrations, excretion rates, ratios)	Glucuronides of androsterone, etiocholanolone, testosterone, epitestosterone, 5α-androstan-3α,17ß-diol, 5ß-androstan-3α,17ß-diol, dihydrotestosterone, tetrahydrocortisol, 11ß-hydroxyandrosterone, 11ß-hydroxyetiocholanolone, pregnandiol, pregnantriol	GC/MSD
Peptide hormones in urine	LH, FSH, HCG	Enzymimmunoassay
Blood parameters	Testosterone, 17α-hydroxyprogesterone (eventually esters of testosterone)	GC/HRMS

USADA 0967

Evaluation

- Statistical evaluation of the steroidprofile parameters and of the peptide hormone values (mean, standard deviation, coefficient of variation) for the determination of the individual reference ranges.
- Comparison of the values of the ES with the values of the suspicious sample.
- Comparison of the values of the ES with population based reference ranges.

Endocrinological study Cologne,

Last name:	Date of birth:
First name:	Sex: male. O female O
Street:	Size [cm]:
City:	Weight: [kg]:
Country:	

Federation:	Professional: O Amateur: O
Major Discipline:	Years of training:
Coach:	Training sessions per week:
Federation doctor:	Training hours per week:

Current training:
Current health status:
Current medication:
Former dope controls: *Date* *City/Event* *Code-Nr.* *Laboratory*
Remarks:

Fig. 2: Questionnaire for the endocrinological study

PRIV. DOZ. DR. WILHELM SCHÄNZER
INSTITUT FÜR BIOCHEMIE
DEUTSCHE SPORTHOCHSCHULE KÖLN
CARL-DIEM-WEG 6
50933 KÖLN

Urine Collection Schedule

Name:

Day/Date	Collection time	Actual collection time	Volume [ml]	Remarks
1. day	7.00			
	10.00			observation
	13.00			
	16.00			observation
	19.00			
	22.00			
2. day	7.00			
	10.00			observation blood sample collectionn
	13.00			
	16.00			observation
	19.00			
	22.00			
3. day	7.00			
	10.00			observation
	13.00			

Fig. 3: Urine collection schedule for the endocrinological study. Several collections take place under observation.

Data evaluation of endocrinological studies or longitudinal studies

In table 7 and 8 are presented the results of an endocrinological study of a male athlete with 12 samples. The coefficients of variation (cv in %) for the presented steroid ratios should be

USADA 0968

within 10 % and 25 % (14,15,16). If we have higher variations, e.g. in longitudinal studies with only 3 or 4 samples, we cannot calculate the subject based refernce range for the TEST/EPIT ratio. For the ratio TEST/LH we observe always variations higher than 20%.

Tab. 7: Results of an ES. Concentrations of endogenous steroids.

#	time	AND [ng/ml]	ETIO [ng/ml]	EPIT [ng/ml]	TEST [ng/ml]
1	10:15	6133	7138	18,6	83,4
2	13:45	2334	2580	6,1	34,5
3	16:05	2917	3252	5,9	35,2
4	19:00	3691	3930	9,6	51,2
5	00:05	2905	3657	9,0	40,1
6	10:45	2286	3103	7,0	45,9
7	12:15	3130	3926	9,0	67,2
8	16:25	4304	4582	13,5	96,3
9	19:15	2164	2389	5,5	36,8
10	22:00	2412	3201	7,3	46,4
11	07:35	2047	2437	5,2	36,1
12	10:45	3400	4525	10,0	75,2

Tab. 8: Results of an ES. Ratios of endogenous steroids and LH values. The ratios TEST/EPIT are corrected with the internal standards d3-TEST/d3-EPIT 6:1 (7). Presented are also the mean, standard deviation (stdev) and coefficient of variation in percent (cv %).

#	time	TEST/ EPIT corr.	AND/ TEST conc	AND/ EPIT conc	AND/ ETIO conc	LH [mIU/ml]	TEST/LH [nmol/IU]
1	10:15	4,6	73,5	330,1	0,9	9,4	30,9
2	13:45	4,7	67,7	382,6	0,9	2,1	57,6
3	16:05	5,6	82,9	494,4	0,9	3,1	39,4
4	19:00	4,5	72,1	386,5	0,9	1,9	91,6
5	00:05	4,7	72,4	322,7	0,8	2,7	51,6
6	10:45	5,9	49,8	326,1	0,7	6,9	23,2
7	12:15	6,9	46,6	347,7	0,8	8,0	29,1
8	16:25	6,8	44,7	318,8	0,9	7,8	42,7
9	19:15	5,8	58,8	392,1	0,9	4,6	27,9
10	22:00	5,7	52,0	330,4	0,8	6,7	24,0
11	07:35	6,1	56,7	392,8	0,8	5,8	21,4
12	10:45	6,9	45,2	340,7	0,8	6,4	40,8
mean		**5,7**	**60,2**	**363,7**	**0,8**	**5,5**	**40,0**
stdev		**0,9**	**13,1**	**50,2**	**0,1**	**2,5**	**19,9**
cv %		**15,9**	**21,7**	**13,8**	**8,9**	**46,2**	**49,7**

From mean and standard deviation (stdev) of the corrected TEST/EPIT ratios we calculate the limits of the subjected based refernce range according to the formula (17):

- upper limit of subject based refernce range = mean + 3 x stdev
- lower limit of subject based refernce range = mean - 3 x stdev

In figure 4 are presented the single TEST/EPIT values of an ES and the corresponding reference limits

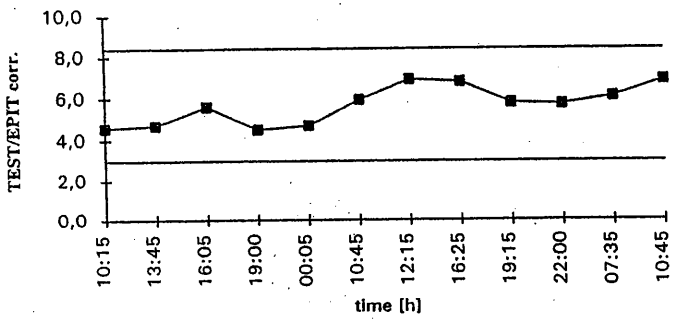


Fig. 4: TEST/EPIT values of an ES and corresponding limits of the subject based reference range (see table 8).

Blood samples in the ES

During the ES we take 10 ml of blood. We take this sample to receive additional TEST related information, e.g. about the ratio TEST/17α - hydroxyprogesteron. The analysis is performed according to the GC/HRMS method described by HORNING et al. (18).
With this blood sample we have also the possibility, to prove an application of TEST during the ES by the detection of TEST-esters in the plasma.

USADA 0969

Ketococonazole Test during the ES

A Ketoconazole test is an effective method for the differentiation between a natural elevated TEST/EPIT and a TEST abuse by an athlete (19). Such a test may be useful to detect a TEST application during the ES. But because of possible health risks by side effects of this drug, we hesitate to perform this test.

Interpretation of the results of an ES or a longitudinal study and recommendation to the federation

If the TEST/EPIT values and the other TEST related ratios of the suspicious samples lie within the subject based reference range and the variations of the ES values, we recommend to give the sample negative. In this case, we have a natural elevated TEST/EPIT ratio. The comparison of the ES values with the population based reference ranges allows to recognize the reason for the increased TEST/EPIT ratio. Most often a low EPIT concentration is responsible for the natural elevated TEST/EPIT ratio. Such a case is presented in tables 7 and 8. A low EPIT case is characterised by the following parameters:

- Concentration of EPIT near or outside the lower limit of the refernce range
- Ratio AND/EPIT near or outside the upper limit of the refernce range
- Ratio AND/TEST near the mean and median of this parameter
- Concentration of TEST near the mean and median of this parameter.

If we have a low EPIT case, we recommend to the federation to give a certification (naturally elevated T/E ratio) to the athlete. The certification should have the following consequences: In case a laboratory shows a "positive " result for TEST related parameters, the Antidoping Commission of the federation should be informed at once and before disciplinary measures be taken against the athlete. The decision positive or negative should be made only after the comparison of the suspicious values with the archived values of the athlete.

References:

1. Donike, M. , Bärwald, K.-R. Klostermann, K., Schänzer, W. Zimmermann, J.: Nachweis von exogenem Testosteron. in: H.Heck, W.Hollmann, H.Liesen, R.Rost (eds.) Sport: Leistung und Gesundheit., Deutscher Ärzte Verlag Köln, (1983) 293.
2. Zimmermann, J.: Untersuchung zum Nachweis von exogenen Gaben von Testosteron. Thesis. Deutsche Sporthochschule Köln. 1986
3. IOC Medical Code, 1995.
4. Donike, M., Ueki, M., Kuroda, Y., Geyer, H., Nolteernsing, E., Rauth, S., Schänzer, W., Schindler, U., Völker, E., Fujisaki, M.: Detection of dihydro-testosterone (DHT) doping - Alterations in the steroid profile and reference ranges for DHT and its 5α-metabolites. J. Sports.Med.Phys.Fitness, 35 (1995) 235-50.
5. Geyer, H., Schänzer, W., Mareck-Engelke, U., Donike, M.: Factors influencing the steroid profile. in: M.Donike, H.Geyer, A.Gotzmann, U.Mareck-Engelke (Eds.) Recent advances in doping analysis (3) Sport und Buch Strauß, Köln (1996) 95-114.
6. Rauth, S.: Referenzbereiche von urinären Steroidkonzentrationen und Steroidquotienten. Ein Beitrag zur Interpretation des Steroidprofils in der Routinedopinganalytik. Dissertation, Deutsche Sporthochschule Köln (1994).
7. Nolteernsting, E., Geyer, H., Mareck-Engelke, U., Schänzer, W., Donike, M.: Standardisation of the T/E-determination by deuterated internal standards. In: M. Donike, H.Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln (1995) 113-120.
8. Geyer, H.: Die gas-chromatographisch/massenspektrometrischeBestimmung von Steroidprofilen im Urin von Athleten. Dissertation, Deutsche Sporthochschule Köln (1990).
9. Kuoppasalmi, K., Leinonen, A., Kajalainen, U.: Detection of exogenous testosterone in doping analysis: methodological aspects. In: Sports medicine and exercice science. Proceedings of the Olympic scientific congress. Eugene, Oregon, 1984.
10. Geyer, H., Mareck-Engelke, U., Schänzer, W., Donike, M.: Simple purification of urine samples for improved detection of anabolic and endogenous steroids. In: M. Donike, H.Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.) Recent advances in doping analysis. Sport und Buch Strauß, Köln (1994) 97-104.
11. Kicman, A.T., Brooks, R.V., Collyer, S.C.et al.: Criteria to indicate testosterone administration. Brit.J.Sports Med. 24 (1990) 253-264.
12. Cowan, D.A., Kicman, A.T., Walker, C.J., Wheeler, M.J.: Effects of administration of human chorionic gonadotrophin on criteria used to assess testosterone administration in athletes. J. of Endocrinology 131 (1991) 147-154.
13. Mareck-Engelke U., Geyer H., Schindler U., Flenker U., Iffland R. and Donike M.: Influence of Ethanol on Steroid Profile Parameters in: M.Donike, H.Geyer, A.Gotzmann, U.Mareck-Engelke (Eds.) Recent advances in doping analysis (3) Sport und Buch Strauß, Köln (1996) 143-156.

USADA 0970

14. Mareck-Engelke, U., Geyer, H., Donike, M: Stability of steroidprofiles (3): Ratios and excretion rates of endogenous steroids in male urines collected over 24 hours. In: M. Donike, H.Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln (1995) 121-134
15. Mareck-Engelke, U., Geyer, H., Donike, M: Stability of steroidprofiles (4): Ratios and excretion rates of endogenous steroids in female urines collected four times over 24 hours. In: M. Donike, H.Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln (1995) 135-156.
16. Nitschke, R.: Steroidprofile und Veränderungen biochemischer Parameter von Hochleistungsradrennfahrern unter extremen Ausdauerbelastungen während zwei Rundfahrten. Dissertation, Deutsche Sporthochschule Köln, 1997.
17. Donike, M., Rauth, S., Mareck-Engelke, U., Geyer, H., Nitschke, R.: Evaluation of longitudinal studies, the determination of subject based reference ranges of the testosterone/ epitestosterone ratio. In: M. Donike, H.Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.) Recent advances in doping analysis. Sport und Buch Strauß, Köln (1994) 33-40.
18. Horning, S., Schänzer, W., Donike, M.: GC/HRMS determination of the serum (plasma) testosterone/17α-hydroxyprogesterone ratio and its significance. In: M. Donike, H.Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln (1995) 51-58.
19. Kicman, A.T., Oftebro, H., Walker, C., Norma, N., Cowan, D.A.: Potential use of ketoconazole in a dynamic endocrine test to differentiate between biological outliers and testosterone use by athletes. Clin. Chem. 39/9 (1993) 1798-1803.

TAB 44

Johncie Wingard

From: Johncie Wingard
Sent: Tuesday, November 28, 2006 10:49 AM
To: 'Ana Reyes'
Subject: RE: URGENT!

Thank you! Please hold and Rich Young will contact Don.

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

From: Ana Reyes [mailto:afreyes@ucla.edu]
Sent: Tuesday, November 28, 2006 10:47 AM
To: Johncie Wingard
Subject: RE: URGENT!

Yes, we still have them.

Ana Reyes
Service/Quality Manager
UCLA Olympic Analytical Laboratory
Main Phone: (310) 825-2635
FAX: (310) 206-9077
E-mail:afreyes@ucla.edu

From: Johncie Wingard [mailto:jwingard@usantidoping.org]
Sent: Tuesday, November 28, 2006 7:33 AM
To: afreyes@ucla.edu
Subject: URGENT!

Ana – could you please see if you still have the B samples for the following:

JSADA # 497104 – UCLA # 9S604
JSADA # - 1501850 - UCLA # 94T03

f so, please **DO NOT DISCARD**. Rich Young will be contacting Don about them. Thank you.

Johncie B. Wingard
Par gal
Jnited States Anti-Doping Agency
330 Quail Lake Loop, Suite 260

Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank you.

Johncie Wingard

om: Johncie Wingard
Sent: Thursday, November 30, 2006 3:24 PM
To: 'direction@lndd.com'
Subject: SENT ON BEHALF OF TRAVIS TYGART

Dear Dr. Ceaurriz,

We understand that the B specimen bottles of UCI Samples 995462, 994203, 994277, 994276, 994075, 994080 and 994171 are still available for additional analysis at LNDD. USADA hereby requests that LNDD analyze these urines using the GC-IRMS method to ascertain whether any of these samples contains exogenous testosterone or its precursors. If practical, we would like these samples to be analyzed at the same time as two additional samples (USADA #497104 and USADA #1501850) which will be forwarded to you shortly by the UCLA laboratory.

If you have any questions or concerns, do not hesitate to contact me.

Sincerely,

Travis Tygart

Johncie B. Wingard
P legal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

hncie Wingard

From: Johncie Wingard
Sent: Friday, December 01, 2006 2:33 PM
To: 'Don (MD) Catlin (dcatlin@ucla.edu)'
Cc: 'Ana Reyes'; 'direction@lndd.com'; 'msekera@ucla.edu'
Subject: SENT ON BEHALF OF TRAVIS TYGART

Dear Don,

Could you please forward the A specimen bottles (if any urine remains) and the B specimen bottles of Samples USADA # 497104 (UCLA # 9S604) and USADA # 1501850 (UCLA # 94T03) to the Paris laboratory? Dr. Ceaurriz will be expecting them. Please feel free to contact Dr. Ceaurriz to coordinate shipment at USADA's expense. I have copied him on this email.

If you have any questions, feel free to contact me.

Sincerely,

Travis Tygart

J ıcie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

ɔhncie Wingard

From: Don Catlin [dcatlin@ucla.edu]
Sent: Friday, December 01, 2006 3:38 PM
To: Johncie Wingard
Subject: RE: SENT ON BEHALF OF TRAVIS TYGART

Travis,
We will send the unopened B. That will suffice for their purpose. We will keep the opened A in case there is some issue that arrises.
Don

From: Johncie Wingard [mailto:jwingard@usantidoping.org]
Sent: Friday, December 01, 2006 1:33 PM
To: dcatlin@ucla.edu
Cc: Ana Reyes; direction@lndd.com; msekera@ucla.edu
Subject: SENT ON BEHALF OF TRAVIS TYGART

Dear Don,

Could you please forward the A specimen bottles (if any urine remains) and the B specimen bottles of Samples USADA # 497104 (UCLA # 9S604) and USADA # 1501850 (UCLA # 94T03) to the Paris laboratory? Dr. Ceaurriz will be expecting them. Please feel free to contact Dr. Ceaurriz to coordinate shipment at USADA's expense. I have copied him on this email.

If you have any questions, feel free to contact me.

Sincerely,

Travis Tygart

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T) 719.785.2032
(F) 719.785.2001
www.usantidoping.org

CONFIDENTIALITY NOTICE - This e-mail transmission, and any documents, files or previous e-mail messages attached to it may contain information that is confidential or legally privileged. If you are not the intended recipient, or a person responsible for delivering it to the intended recipient, you are hereby notified that you must not read this transmission and that any disclosure, copying, printing, distribution or use of any of the information contained in or attached to this transmission is STRICTLY PROHIBITED. If you have received this transmission in error, please immediately notify the sender by telephone or return e-mail and delete the original transmission and its attachments without reading or saving in any manner. Thank

afld

Agence française de lutte contre le dopage

Date : 14/12/06

De la part de : Philippe DAUTRY,
Secrétaire général
Agence française de lutte contre le dopage (AFLD)

Coordonnées : 01 40 62 76 77

Destinataire : M. Tygart

Télécopie : 00.1.719.785.2001

Nombre de pages : 1+ 1

Dear Mr. Tygart,

this letter is in answer to your demand to Mr. de Ceaurriz. Don't hesitate to contact me.

Best regards,

Ph. Dautry

00.33.1.40.62.76.76

afld

Agence française de lutte contre le dopage

Le Président

Paris, le 14 décembre 2006,

Cher Monsieur Tygart,

M. Jacques de Ceaurriz, directeur des analyses de l'Agence française de lutte contre le dopage (AFLD), m'a fait part de votre télécopie du 30 novembre 2006, dans laquelle vous lui demandez de procéder, au département de l'Agence, à l'analyse de plusieurs échantillons urinaires B (995462, 994203, 994277, 994276, 994075, 994080 et 994171).

Le directeur des analyses de l'Agence a indiqué la faisabilité technique de ces analyses, sous certaines réserves.

Sur le fond, je vous confirme la possibilité pour l'AFLD de procéder aux analyses que vous souhaitez sous les réserves suivantes :

- ces analyses d'échantillons B devraient être réalisées après information du sportif concerné ;

- si ce dernier le demande, elles devraient être faites en présence d'experts, conformément aux règles applicables aux secondes analyses sur les échantillons B,

- la charge financière de ces analyses ne devrait pas être entièrement supportée par le département des analyses de l'AFLD, compte tenu de la durée prévisible de ces tests.

Je vous saurais gré de me faire savoir si ces conditions vous agréent, et vous prie d'agréer, Cher Monsieur, l'expression de mes sentiments distingués.

Pierre BORDRY

Monsieur Travis TYGART
United states anti-doping agency
1330 Quai Lake loop, suite 260
Colorado springs CO 80906
United States of America

afld
agence française de lutte contre le dopage
Département des Analyses

Châtenay-Malabry, the 27th December 2006

TRANSMISSION DE TELECOPIE

Expéditeur :	**Destinataire** :
J. de CEAURRIZ Directeur du Département des Analyses **Tél** : +33 (0) 1.46.60.28.69 **Fax** : +33 (0) 1.46.60.30.17 **e-mail** : Analyses@afld.fr	Travis T. Tygart **Organisme** : USADA **Fax** : 00.1.719.785.2001

Nombre de pages y compris celle-ci : 2

Dear Travis Tygart,

Please, find here the proposal of our laboratory for the IRMS analysis of samples n° 995462 B, n° 994203 B, n° 994277 B, n° 994276 B, n° 994075 B, n° 994080 B, n° 994171 B, from Tour de France 2006 and IRMS analysis of the two additional B samples from USADA n° 497104 B = UCLA 9S604 and n° 1501850 B = UCLA 94T03.
The B sample analysis will be performed from January 8, 2007 to January 25, 2007. A more detailed program was attached to this fax letter.

Regarding the presence of an analytical expert for looking at the data stored on computer, the most convenient days will be January 15 and 16, 2007 as well as January 22 and 23, 2007.

Yours Sincerely,

J. de CEAURRIZ

Second Week of Janua. , (January 8 to 12, 2007)

	January 8 Monday	January 9 Tuesday	January 10 Wednesday	January 11 Thursday	January 12 Friday
Opening at 9h00 a.m	B 995462 B 994203	B 994277 B 994276			
Sample extraction and derivatization	B 995462 B 994203	B 995462 B 994203 B 994277 B 994276	B 994277 B 994276		
GC-MS and IRMS analysis			B 995462 B 994203	B 994277 B 994276	
Result available				B 995462 B 994203	B 994277 B 994276

Third Week of January (January 15 to 19, 2007)

	January 15 Monday	January 16 Tuesday	January 17 Wednesday	January 18 Thursday	January 19 Friday
Opening at 9h00 a.m	UCLA 9S604 B 994075	B 994080			
Sample extraction and derivatization	UCLA 9S604 B 994075	UCLA 9S604 B 994075 B 994080	B 994080		
GC-MS and IRMS analysis			UCLA 9S604 B 994075	B 994080	
Result available				UCLA 9S604 B 994075	B 994080

Fourth Week of January (January 22 to 26, 2007)

	January 22 Monday	January 23 Tuesday	January 24 Wednesday	January 25 Thursday	January 26 Friday
Opening at 9h00 a.m	B 994171 UCLA 94T03				
Sample extraction and derivatization	B 994171 UCLA 94T03	B 994171 UCLA 94T03			
GC-MS and IRMS analysis			B 994171 UCLA 94T03		
Result available				B 994171 UCLA 94T03	

USADA 0979

Lance . . . Jan 27-Dec-06 12:05; Page 2/2

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

VIA FACSIMILE and VIA EMAIL

Jacques de Ceaurriz
Directeur de Département des Analyses
AFLD
143, Avenue Roger Salengro
92290 Châtenay-Malabry

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

Dear Dr. de Ceaurriz:

We have notified the athlete's counsel of the schedule for the IRMS analysis of the samples to be performed from January 8, 2007 to January 25, 2007. We have asked that we be notified no later than January 4, 2007, if a representative will be in attendance. We will notify you once we have been informed by the athlete whether he will have a representative in attendance.

Please proceed with the analysis as outlined on your scheduling document that you sent to me and that I have attached to this letter.

Thank you again for your assistance.

Sincerely,

Travis T. Tygart
General Counsel

United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260 Colorado Springs, CO 80906 ▪ Tel: 719.785.2000 ▪ Fax: 719.785.2001

Second Week of January (January 8 to 12, 2007)

	January 8 Monday	January 9 Tuesday	January 10 Wednesday	January 11 Thursday	January 12 Friday
Opening at 9h00 a.m	B 995462 B 994203	B 994277 B 994276			
Sample extraction and derivatization	B 995462 B 994203	B 995462 B 994203 B 994277 B 994276	B 994277 B 994276		
GC-MS and IRMS analysis			B 995462 B 994203	B 994277 B 994276	
Result available				B 995462 B 994203	B 994277 B 994276

Third Week of January (January 15 to 19, 2007)

	January 15 Monday	January 16 Tuesday	January 17 Wednesday	January 18 Thursday	January 19 Friday
Opening at 9h00 a.m	UCLA 9S604 B 994075	B 994080			
Sample extraction and derivatization	UCLA 9S604 B 994075	UCLA 9S604 B 994075 B 994080	B 994080		
GC-MS and IRMS analysis			UCLA 9S604 B 994075	B 994080	
Result available				UCLA 9S604 B 994075	B 994080

Fourth Week of January (January 22 to 26, 2007)

	January 22 Monday	January 23 Tuesday	January 24 Wednesday	January 25 Thursday	January 26 Friday
Opening at 9h00 a.m	B 994171 UCLA 94T03				
Sample extraction and derivatization	B 994171 UCLA 94T03	B 994171 UCLA 94T03			
GC-MS and IRMS analysis			B 994171 UCLA 94T03		
Result available				B 994171 UCLA 94T03	

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

VIA FACSIMILE

December 27, 2006

Howard L. Jacobs, Esq.
5210 Lewis Road, Suite 5
Agoura Hills, CA 91301

Re: USADA v. Floyd Landis
AAA No. 30 190 00847 06

Dear Howard:

I am writing to notify you that the WADA-accredited laboratory at Châtenay-Malabry, France will be analyzing samples provided by Mr. Floyd Landis during the Tour De France and two samples collected subsequent to the Tour De France by USADA, using the IRMS analysis commencing on January 8, 2007.

Also, you are welcome to have an observer present to view the opening and analysis of these samples if you desire. Given the number of samples and the laboratory's schedule, it is anticipated that the analysis will conclude on January 25, 2007. I have enclosed a schedule outlining the anticipated steps in the analytical process with the corresponding sample numbers that will be analyzed by the French laboratory.

If you intend to have an observer present for all or any portion of the process, please inform me and provide that person's name as soon as possible but no later than **January 4, 2007** so that I may inform the laboratory of the observer's attendance.

Additionally, it was my understanding based on our telephone conversation of last week concerning your discovery requests that you were going to inform me whether or not you desired access to the computer data from sample #995474. Please inform me when you have come to a decision on this matter.

If you have any questions, please do not hesitate to contact me directly.

Sincerely,

Travis T. Tygart
General Counsel

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260, Colorado Springs, CO 80906 ■ Tel: 719.785.2000 ■ Fax: 719.785.2001

Second Week of January (January 8 to 12, 2007)

	January 8 Monday	January 9 Tuesday	January 10 Wednesday	January 11 Thursday	January 12 Friday
Opening at 9h00 a.m	B 995462 B 994203	B 994277 B 994276			
Sample extraction and derivatization	B 995462 B 994203	B 995462 B 994203 B 994277 B 994276	B 994277 B 994276		
GC-MS and IRMS analysis			B 995462 B 994203	B 994277 B 994276	
Result available				B 995462 B 994203	B 994277 B 994276

Third Week of January (January 15 to 19, 2007)

	January 15 Monday	January 16 Tuesday	January 17 Wednesday	January 18 Thursday	January 19 Friday
pening at 9h00 a.m	UCLA 9S604 B 994075	B 994080			
ample extraction and derivatization	UCLA 9S604 B 994075	UCLA 9S604 B 994075 B 994080	B 994080		
C-MS and IRMS analysis			UCLA 9S604 B 994075	B 994080	
esult available				UCLA 9S604 B 994075	B 994080

Fourth Week of January (January 22 to 26, 2007)

	January 22 Monday	January 23 Tuesday	January 24 Wednesday	January 25 Thursday	January 26 Friday
pening at 9h00 a.m	B 994171 UCLA 94T03				
mple extraction and derivatization	B 994171 UCLA 94T03	B 994171 UCLA 94T03			
-MS and IRMS analysis			B 994171 UCLA 94T03		
sult available				B 994171 UCLA 94T03	

GIBSON, DUNN & CRUTCHER LLP
LAWYERS
A REGISTERED LIMITED LIABILITY PARTNERSHIP
INCLUDING PROFESSIONAL CORPORATIONS

333 South Grand Avenue Los Angeles, California 90071-3197
(213) 229-7000
www.gibsondunn.com

MSuh@gibsondunn.com

December 29, 2006

Direct Dial
(213) 229-7260
Fax No.
(213) 229-6260

Client No.
T54563-00001

VIA FACSIMILE AND
REGULAR U.S. MAIL

Travis T. Tygart
Senior Managing Director and General Counsel
U.S. Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906-4651

Re: *USADA v. Floyd Landis*
AAA Case No. 30 190 00847 06

Dear Mr. Tygart:

Thank you for our telephone conversation today. As I indicated to you today, we have recently been retained to defend Floyd Landis in conjunction with the allegations arising from the 2006 Tour de France. We look forward to working with you on this matter. As Howard Jacobs is co-counsel with us, please copy him on all of our communications.

The immediate concern that we have in conjunction with this litigation revolves around your letter dated December 27, 2006 indicating that the Labaratoire National de Dopistage du Dopage ("LNDD") will test other specimens collected from Mr. Landis during and after the 2006 Tour de France. It is clear that none of the initial tests of those very same specimens taken from Mr. Landis has resulted in any finding that any prohibited substance was detected. You have indicated to me that the purpose of this series of retests is to support the preparation and trial of the allegations arising from the Stage 17 test. You have not committed that any of these retests will be or will not be used to support any additional cases or allegations against Mr. Landis. Lastly, you indicated that (1) you requested LNDD to perform these retests; (2) LNDD has agreed to perform the retests on the schedule attached to your December 27, 2006 letter; and (3) the UCLA laboratory has cooperated by sending the out-of-competition samples to LNDD so that the retests can be performed on the schedule attached to your December 27, 2006 letter. The

retesting that you propose in your December 27, 2006 letter thus violates a number of the applicable rules and protocols in athlete drug testing, including, but not limited to, those promulgated by the World Anti Doping Association ("WADA"). We therefore demand that you cease this retesting procedure. Please direct a request to LNDD requesting that no further tests on the described samples in your December 27, 2006 letter be performed and copy me on that correspondence.

As we discussed, I will call you on Monday, January 1, 2007 so that we may continue our discussion with respect to these and other unresolved issues this case.

Thank you in advance for your cooperation in conjunction with the foregoing.

Sincerely,

Maurice M. Suh

MMS/td
cc: Howard Jacobs

100139895_1.DOC

GIBSON, DUNN & CRUTCHER LLP
LAWYERS
A REGISTERED LIMITED LIABILITY PARTNERSHIP
INCLUDING PROFESSIONAL CORPORATIONS

333 South Grand Avenue Los Angeles, California 90071-3197
(213) 229-7000
www.gibsondunn.com

MSuh@gibsondunn.com

January 2, 2007

Direct Dial
(213) 229-7260

Fax No.
(213) 229-6260

Client No.
T54563-00001

VIA FACSIMILE AND
REGULAR U.S. MAIL

Travis T. Tygart
Senior Managing Director and General Counsel
U.S. Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906-4651

Re: *USADA v. Floyd Landis, AAA Case No. 30 190 00847 06*
Illegal Testing of B Samples

Dear Mr. Tygart:

I called you today in an attempt to come to a resolution with respect to the proposed retesting of samples taken during the 2006 Tour de France and the retesting of out-of-competition samples as set forth in your December 27, 2006 letter. In an effort to avoid unnecessary litigation with respect to this matter, I reiterate my earlier demand that you immediately (1) cease this illegal retesting procedure and (2) direct a request to the Labaratoire National du Dopistage ("LNDD") to cease this illegal retesting procedure with a copy of that request to me. Reserving all of our arguments with respect to the proposed illegal retesting, it is clear that your proposal violates provisions of the UCI Anti-Doping Rules, the World Anti-Doping Agency ("WADA") International Standard for Laboratories, the USADA protocol and the WADA Results Management Guidelines, among others.

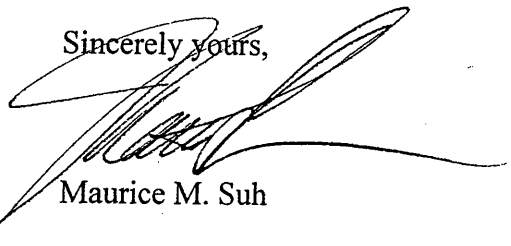
What is at issue here is whether sufficient evidence exists to find Mr. Landis had the presence of exogenous testosterone within his system in the test administered after Stage 17 of the 2006 Tour de France. The violation of these foregoing rules is even more troubling given your described intended use of any potential adverse results of that illegal retesting, which is to bolster the allegations contained in the instant case. In his entire racing career, Mr. Landis has

received notice of no other doping violation and has never been informed of any positive test result aside from the one currently at issue.

It thus appears that the illegal testing of the samples that you propose is designed to inflict irreparable injury on Mr. Landis by (1) smearing his reputation, (2) invading his privacy and (3) destroying evidence that may later on be valuable with respect to this case. Please be advised that we intend to hold you responsible for each of these harms.

Failure to comply with our request to desist in the illegal testing described in your correspondence of December 27, 2006 by January 5, 2007 will force us to take appropriate action to protect Mr. Landis' interests.

Sincerely yours,



Maurice M. Suh

MMS/td

cc: Dr. Jacques de Ceaurriz, Director
Afld - Agence Fran
çaise de Lutte Contre le Dopage
Département des Analyses
143, avenue Roger-Salengro
F - 92290 Châtenay-Malabry
France
Tel.: (33.1) 46 60 28 69
Fax: (33.1) 46 60 30 17
E-mail: Direction@lndd.com

100140793_1.DOC

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

Via Facsimile

January 3, 2007

Maurice M. Suh
Gibson, Dunn & Crutcher LLP
333 South Grand Avenue
Los Angeles, CA 90071-3197

Re: USADA v. Floyd Landis
AAA Case No. 30 190 00847 06

Dear Mr. Suh:

I am in receipt of your correspondence dated January 2, 2007 regarding the above-referenced mater. First, as I explained to you and Mr. Jacobs during our telephone conversation from the early evening of December 29, 2006, because I have received inconsistent messages from you and Mr. Jacobs, I need clarification regarding who is acting as lead counsel for Mr. Landis in this matter, so that I may respond appropriately.

As you are aware, on December 27, 2006 I sent a letter to Mr. Jacobs setting forth a detailed schedule of IRMS testing of several of Mr. Landis's samples and providing Mr. Landis an opportunity to have an expert present during that testing. I then spoke with Mr. Jacobs on the morning of December 29, 2006. Mr. Jacobs raised no objection to the tests, but instead asked if the testing could be done in a shorter period of time and indicated that he would let me know which expert Mr. Landis would ask to be in attendance. He also indicated that he may call later that day with a scheduling question.

As you know, I never received a call regarding the scheduling questions. Instead, I received an introductory call from you where you made a number of demands regarding discovery and answers to various questions. I explained to you that I was in the middle of my three year old son's birthday party, but that I would be happy to address any reasonable concerns. We agreed that you would send me an email outlining the issues you wanted to discuss and that you would call me on January 1, 2007 to further the discussion.

Despite our agreement, you never sent an email or called me as outlined above. I followed up with Mr. Jacobs on January 1, informing him that I had not received the e-mail and that once you did send the e-mail we could set up a conference call to discuss the issues. He indicated that he would speak with you about this.

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

ate Hendrickson Borg, CHMM
ecretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

Instead, I come to the office today, after directly informing you that our offices were closed until today and that I would be out, and find two letters from you dated December 29, 2006 and January 2, 2007 neither of which accurately reflect my conversation with you and Mr. Jacobs of December 29, 2006 or my conversation with Mr. Jacobs of January 1, 2007. I am more than a little dismayed that you have already decided to resort to threats of litigation rather than make a genuine effort to understand the process and discuss any potential issues. I sincerely hope that this is not an indication of the style you intend to employ as we continue toward resolution of this matter.

As I have consistently indicated from the beginning, I am perfectly willing to enter into a reasonable dialogue about any concerns or questions that you have. What I am unwilling to do is waste my resources responding to letters that mischaracterize previous conversations. Further, I would like to remove the possibility for continuing confusion by having Mr. Landis confirm which of his co-counsel will be taking the lead in this matter so that I do not spend additional time engaging in separate conversations with co-counsel who do not seem to be on the same page. Accordingly, please indicate to me in writing who is the lead counsel on this matter, so that I may direct all communication, including telephone calls, to the appropriate representative. If you are taking the lead on this matter, please provide me all of your contact information including your mobile phone if you do not mind me contacting you there.

Once this confusion has been cleared-up, I will be in contact with that person concerning the matters previously discussed.

Also, please disclose you and your firm's involvement in this matter to the American Arbitration Association and the arbitrators in this case in order to clear any potential conflicts as expeditiously as possible. Please copy me on your appearance notice.

For your future use, please send all communication directly to me. You are not authorized to send communications to persons or entities that you are aware will be witnesses in this matter including the French laboratory.

Sincerely,

Travis T. Tygart
General Counsel

cc: Howard L. Jacobs, Esq.

GIBSON, DUNN & CRUTCHER LLP

LAWYERS

A REGISTERED LIMITED LIABILITY PARTNERSHIP
INCLUDING PROFESSIONAL CORPORATIONS

333 South Grand Avenue Los Angeles, California 90071-3197
(213) 229-7000
www.gibsondunn.com

MSuh@gibsondunn.com

January 3, 2007

Direct Dial
(213) 229-7260
Fax No.
(213) 229-6260

Client No.
T54563-00001

VIA E-MAIL, FAX AND REGULAR U.S. MAIL

Travis T. Tygart
Senior Managing Director and General Counsel
U.S. Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, Colorado 80906-4651

Re: *USADA v. Floyd Landis, AAA Case No. 30 190 00847 06*
Illegal Testing of B Samples

Dear Mr. Tygart:

This is in response to your correspondence dated January 3, 2007. I am lead counsel in this matter. You may direct all correspondence to me, with a copy of that correspondence to Mr. Jacobs.

In your January 3 letter, you did not indicate a response to our demand that the retesting be halted. As a result, we are now, pursuant to Central District Local Rule 7-19.1, providing you with notice that (1) we intend to file a Complaint in the Central District of California on tomorrow, Thursday, January 4, 2007, naming the United States Anti-Doping Agency as a defendant and (2) we intend on filing an application for Temporary Restraining Order to prevent the illegal retesting on Friday, January 5, 2007. Please let me know if you oppose the application or if you intend to be present when the foregoing application is presented to the Court.

Please be aware that we consider the illegal retesting to constitute spoliation of evidence. Because the testing of the B Samples will use up the remainder of the samples, and because the testing procedures and protocol of the French laboratory are at issue in this case, we put you on notice that your proposed illegal retesting will destroy valuable evidence in this case.

As for the balance of the misrepresentations contained in your letter, suffice it to say that Mr. Jacobs and I both disagree with your characterizations of our previous conversations. Notwithstanding that, please let me reiterate that we wish to resolve the outstanding matter in this litigation in as cordial and mutually agreeable manner as possible. In that vein, for ease of communication, I suggest that we could agree that email correspondence is equivalent to signed and delivered correspondence.

Sincerely yours,

Maurice M. Suh

MMS/td
cc: Howard L. Jacobs

100141633_1.DOC

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

Via Facsimile

January 4, 2007

Maurice M. Suh
Gibson, Dunn & Crutcher LLP
333 South Grand Avenue
Los Angeles, CA 90071-3197

Re: USADA v. Floyd Landis
AAA Case No. 30 190 00847 06

Dear Mr. Suh:

This letter responds to your correspondence dated January 3, 2007 regarding the above-referenced matter. Thank you for your clarification that you are acting as lead counsel in this matter.

With respect to your threat to initiate litigation, USADA maintains that your objections to the testing of Mr. Landis's samples are without merit. Further, any action you file in the Central District of California is also without merit because that court does not have jurisdiction over this matter. Instead, if you insist on pursuing your baseless objection, the appropriate forum for that objection to be heard is before the arbitration Panel that has been appointed to hear this matter.

At this point your failure to enter an appearance with the American Arbitration Association in this matter is delaying the final confirmation of the Panel that has been selected pursuant to the rules. For that reason, USADA is willing to delay, for a reasonable time, our request to have the samples analyzed in order to give you additional time to enter your appearance and raise your objection before the Panel. USADA fully expects that after considering the merits of your arguments the Panel will overrule your objection and the testing will proceed. Any delay caused by this process will be solely attributable to your decision to pursue your baseless objection.

Please inform me at your earliest convenience if you accept our offer to temporarily delay the testing pending your submission of your objection to the Panel in this matter. Should you decide instead to go forward with a frivolous legal action, USADA will oppose your application and William Swank, from the Los Angeles office of Holme Roberts & Owen, will serve as

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

ɔte Hendrickson Borg, CHMM
ɔecretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

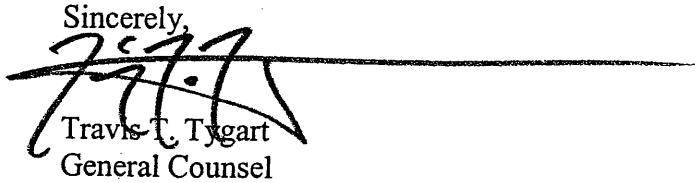
Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

our counsel. Please continue to direct all correspondence regarding this matter to me and please also copy Mr. Swank as a courtesy.

Sincerely,



Travis T. Tygart
General Counsel

cc: William Swank, Esq.
Howard L. Jacobs, Esq.

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

Via Facsimile

January 4, 2007

Maurice M. Suh
Gibson, Dunn & Crutcher LLP
333 South Grand Avenue
Los Angeles, CA 90071-3197

Re: USADA v. Floyd Landis
AAA Case No. 30 190 00847 06

Dear Mr. Suh:

This letter confirms our discussion by telephone this afternoon that you have requested that I temporary postpone my request for the testing of Mr. Landis's samples at the Laboratoire National de Dépistage du Dopage ("LNDD"), which is currently scheduled to commence on January 8, 2007.

As we discussed, you have requested additional time in order to consider issues related to the testing, potentially arrange for an expert to be present at the laboratory and to allow you and I to discuss any other issues related to the testing. Accordingly, we are temporarily postponing our request for the testing of these samples. I will copy you on my letter to LNDD temporarily withdrawing our request that these samples be tested. I am not aware of any other requests from entities other than USADA for testing of these samples but as you know, we do not control what any other entity does with respect to requesting the testing of these samples.

Lastly, as we discussed, please copy me on your entry of appearance with the American Arbitration Association so that we can finalize the arbitration panel as expeditiously as possible.

I look forward to working with you to resolve any remaining issues related to this matter.

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

ıte Hendrickson Borg, CHMM
ɔecretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

Sincerely,

Travis T. Tygart
General Counsel

cc: William Swank, Esq.
Howard L. Jacobs, Esq.

afld
agence française de lutte contre le dopage
Département des Analyses

Châtenay-Malabry, the 4th of January 2007

TRANSMISSION DE TELECOPIE

Expéditeur :

Aurélie Laurent
Quality assistant
Agence française de lutte contre le Dopage
Département des Analyses

Tél : +33 (0) 1.46.60.28.69
Fax : +33 (0) 1.46.60.30.17
e-mail : Analyses@afld.fr

Destinataire :

Travis T. Tygart

Organisme :

USADA

Fax : 00.1.719.785.2001

Nombre de pages y compris celle-ci : 1

Dear Mr Tygart,

We have received a copy of a fax letter from Mr Suh to USADA, indicating that Mr Landis is opposed to the retesting of his samples.

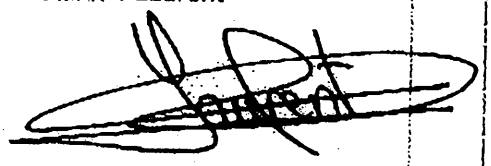
Could you please, confirm us that we must perform the retesting of these samples (we had planned to begin on next Monday).

Please, not that we need a quick answer.

Thank you for your comprehension.

Yours sincerely,

For the Director, Mr J. de Ceaurriz
Aurélie Laurent



USADA 0996

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

VIA FACSIMILE

Jacques de Ceaurriz
Directeur de Département des Analyses
AFLD
143, Avenue Roger Salengro
92290 Châtenay-Malabry

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

Dear Dr. de Ceaurriz:

This is a follow up to our request for the IRMS analysis of the samples scheduled to commence January 8, 2007.

I am writing to alert you that the schedule for this testing has been temporarily postponed. I will contact you concerning revised dates for this testing once we have more information. Accordingly, please confirm your receipt of this request not to commence testing as previously scheduled.

Thank you again for your assistance and we apologize for any inconvenience this has caused you and your staff.

Sincerely,

Travis T. Tygart
General Counsel

cc: Maurice Suh

United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260, Colorado Springs, CO 80906 ■ Tel: 719.785.2000 ■ Fax: 719.785.2001

afld
agence française de lutte contre le dopage
Département des Analyses

Châtenay-Malabry, the 5th of January 2007

TRANSMISSION DE TELECOPIE

Expéditeur :	**Destinataire** :
Aurélie Laurent Quality assistant Agence française de lutte contre le Dopage Département des Analyses **Tél** : +33 (0) 1.46.60.28.69 **Fax** : +33 (0) 1.46.60.30.17 **e-mail** : Analyses@afld.fr	Travis T. Tygart **Organisme** : USADA **Fax** : 00.1.719.785.2001

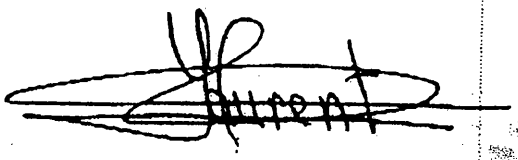
Nombre de pages y compris celle-ci : 1

Dear Mr Tygart,

We have received your fax letter and we are waiting for new dates for retesting samples.

Yours sincerely,

For the Director, Mr J. de Ceaurriz
Aurélie Laurent



afld
agence française de lutte contre le dopage
Département des Analyses

Châtenay-Malabry, the 25th January 2007

TRANSMISSION DE TELECOPIE

Expéditeur :	**Destinataire** :
J. de CEAURRIZ Directeur du Département des Analyses **Tél** : +33 (0) 1.46.60.28.69 **Fax** : +33 (0) 1.46.60.30.17 **e-mail** : Analyses@afld.fr	Travis T. Tygart **Organisme** : USADA **Fax** : 00.1.719.785.2001

Nombre de pages y compris celle-ci : 2

Dear Travis Tygart,

Please, find here a new proposal from our laboratory for the IRMS analysis of samples n° 995462 B, n° 994203 B, n° 994277 B, n° 994276 B, n° 994075 B, n° 994080 B, n° 994171 B, from Tour de France 2006 and IRMS analysis of the two additional B samples from USADA n° 497104 B = UCLA 9S604 and n° 1501850 B = UCLA 94T03 in February 2007

The B analysis may be performed from February 5 to February 23, 2007. A more detailed program was attached to this fax letter.

Yours Sincerely,

J. de CEAURRIZ

February 5 to 9, 2007

	February 5 Monday	February 6 Tuesday	February 7 Wednesday	February 8 Thursday	February 9 Friday
ening at 9h00 a.m	B 995462 B 994203	B 994277 B 994276			
ple extraction and derivatization	B 995462 B 994203	B 995462 B 994203 B 994277 B 994276	B 994277 B 994276		
MS and IRMS analysis			B 995462 B 994203	B 994277 B 994276	
ult available				B 995462 B 994203	B 994277 B 994276

February 12 to 16, 2007

	February 12 Monday	February 13 Tuesday	February 14 Wednesday	February 15 Thursday	February 16 Friday
ing at 9h00 a.m	UCLA 9S604 B 994075	B 994080			
le extraction and derivatization	UCLA 9S604 B 994075	UCLA 9S604 B 994075 B 994080	B 994080		
S and IRMS analysis			UCLA 9S604 B 994075	B 994080	
available				UCLA 9S604 B 994075	B 994080

February 19 to 23, 2007

	February 19 Monday	February 20 Tuesday	February 21 Wednesday	February 22 Thursday	February 23 Friday
g at 9h00 a.m	B 994171 UCLA 94T03				
e extraction and derivatization	B 994171 UCLA 94T03	B 994171 UCLA 94T03			
and IRMS analysis			B 994171 UCLA 94T03		
available				B 994171 UCLA 94T03	

Laser Jet 3100; 25-Jan-07 11:45; Page 2/2

USADA
U.S. Anti-Doping Agency
Integrity. Health. Sport.

VIA FACSIMILE

January 31, 2007

Jacques de Ceaurriz
Directeur de Département des Analyses
AFLD
143, Avenue Roger Salengro
92290 Châtenay-Malabry

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

Dear Dr. de Ceaurriz:

I am in receipt of your letter dated January 25, 2007 regarding the IRMS analysis of various samples scheduled to commence February 5, 2007.

I am writing to request that you temporarily postpone this testing. I will contact you concerning rescheduling the testing once I have more information. Accordingly, please confirm your receipt of this request not to commence testing as previously scheduled.

Thank you again for your patience and I apologize for any inconvenience this has caused you and your staff.

Sincerely,

Travis T. Tygart
General Counsel

cc: Maurice Suh, Esq.

USADA 1001

afld
agence française de lutte contre le dopage
Département des Analyses

Châtenay-Malabry, the 1st February, 2007

TRANSMISSION DE TELECOPIE

Expéditeur :

J. de CEAURRIZ
Directeur du Département des Analyses

Tél : +33 (0) 1.46.60.28.69
Fax : +33 (0) 1.46.60.30.17
e-mail : Analyses@afld.fr

Destinataire :

Travis T. Tygart
Organisme :

USADA

Fax : 00.1.719.785.2001

Nombre de pages y compris celle-ci : 2

Dear Travis Tygart,

I confirm the receipt of your request to temporarily postpone testing previously scheduled to commence February 5, 2007.

We are waiting your decision before rescheduling the testing.

Yours Sincerely,

J. de CEAURRIZ

TAB 45

Johncie Wingard

From: Johncie Wingard
Sent: Friday, December 01, 2006 3:36 PM
To: 'direction@lndd.com'
Cc: 'p.dautry@afld.fr'; 'philippe.verbiest@uci.ch'; 'Olivier.Niggli@wada-ama.org'

Dear Dr. Ceaurriz,

As you know, USADA is proceeding with an anti-doping rule violation case against Floyd Landis on behalf of UCI and USA Cycling arising out of the positive test at the Tour de France. We would greatly appreciate it if you could provide us with a brief summary of your responses to the arguments raised by Dr. Baker in his PowerPoint presentation which can be found on Landis's internet website.

Thank you for your assistance.

Travis Tygart

Johncie B. Wingard
Paralegal
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906
(T 719.785.2032
(F) 719.785.2001
www.usantidoping.org

afld
agence française de lutte contre le dopage
Département des Analyses

Châtenay-Malabry, le 5th december 2006

TRANSMISSION DE TELECOPIE

Expéditeur :	Destinataire :
J. de CEAURRIZ Directeur du département de l'afld Tél : +33 (0) 1.46.60.28.69 Fax : +33 (0) 1.46.60.30.17 e-mail : direction@lndd.com	Travis TYGART Organisme : USADA Fax : 00 719.785.2001

Nombre de pages y compris celle-ci :4

Dear Travis Tygart,

Please, find here the French version of the response of our laboratory on the argument raised by Dr. Baker on his PowerPoint presentation (document dated on 2006/10/11).

This response was made at the request of the French Anti-doping Agency (afld) to clear the matter up and confidentially transmitted to this national authority.

Since that time, a new document date on 2006/11/18 was prepared by Dr. Baker. It contained new argument, especially those dealing with the time-dependent interlaboratory differences in the critical isotopic depletion values between UCLA, Sydney and Paris laboratories. To my knowledge, UCLA and Sydney criteria were based on the mean, ±3SD isotopic depletion values from a control subpopulation and did not need any further modulation by uncertainty. The expression of results from Paris was based on the recent WADA criterion (isotopic depletion value of 3) modulated by the uncertainty of 0.8 ‰.

To my opinion, the two different manners in expressing the isotopic depletion results were very close and the 13Cδ‰ depletion value of 3 recommended by WADA can be regarded as the result of a successful harmonization contrary to what Dr. Baker had declared in this new document.

Best regards,

J. de CEAURRIZ

afld
agence française de lutte contre le dopage
Département des Analyses

Commentaires du département des analyses de l'afld sur l'argumentaire de F. Landis

Préambule :

L'argumentaire de F. Landis a été préparé par Arnie Baker et figure dans un document de 26 pages qui a été communiqué au département des analyses de l'afld par l'AMA (O. Rabin) et l'UCI (M. Zorzoli).

1/ Erreurs dactylographiques et manuscrites

Page 3 de l'argumentaire de F. Landis, il est fait référence à l'enregistrement qui figure à la page 60 du dossier de contre-analyse du laboratoire. Il est exact que cet enregistrement qui récapitule une partie des résultats présente deux erreurs dactylographiques au niveau de la numérotation de l'échantillon 478/07_994474 au lieu de 178/07_995474.
Les conséquences de cette erreur sont minimes et peuvent être appréciées au regard des résultats associés dans ce même enregistrement aux fichiers informatiques 178 0774A, 178 0774B et 178 0774C qui récapitulent les résultats bruts des pages 49 à 54 du même dossier où figurent la bonne numérotation de l'échantillon et l'identification des fichiers informatiques correspondants. Il n'y a donc aucune perte de la filiation entre les résultats et le numéro d'identification de l'échantillon en dépit de l'erreur dactylographique.

Pages 4 et 5 de l'argumentaire de F. Landis, il est fait référence à l'enregistrement qui figure à la page 5 du dossier d'analyse de l'échantillon A du laboratoire et à une correction manuscrite instantanée par superposition du chiffre 5 dans le numéro de l'échantillon 995474.

Page 6 de l'argumentation de F. Landis, il est fait référence à la page 21 du dossier analytique de l'échantillon A où il existe une difficulté à lire le dernier chiffre 4 du numéro manuscrit de l'échantillon 995474.

Page 7 de l'argumentation de F. Landis, il est fait référence à la page 4 (verso) de l'enregistrement figurant dans le dossier analytique de l'échantillon A et d'une erreur manuscrite du numéro de l'échantillon 995475 au lieu de 995474.
Les conséquences des deux erreurs manuscrites des pages 4 et 5 du dossier analytique de l'échantillon A relatives au numéro 995474 sont à apprécier au regard de l'ensemble du dossier analytique et du maintien d'une filiation entre le n° 995474 et les résultats analytiques (par exemple pages 4 (recto), 49 et 178 du document analytique de l'échantillon A).

www.afld.fr | 143, avenue Roger Salengro 92290 Châtenay-Malabry | Tél : +33 (0)1 46 60 28 68 | Fax : +33 (0)1 46 60 30 17

afld
agence française de lutte contre le dopage
Département des Analyses

2/ Contamination / dégradation de l'échantillon 995474 B

Il est fait référence à la page 8 de l'argumentaire de F. Landis d'une dégradation de l'échantillon B sur la base de l'existence d'un pourcentage d'épitestostérone libre > à 5 % (7,7 %) à partir des résultats figurant pages 55 et 56 du dossier de contre-analyse. La valeur de 0.44 ng/mL calculée par l'ordinateur correspond à un signal d'épitestostérone qui appartient au bruit de fond et se situe au dessous de la limite de quantification de ce paramètre. Ce résultat est donc ininterprétable et le calcul du pourcentage d'épitestostérone libre n'a pas de sens. L'argumentaire n'évoque pas la concentration de testostérone libre inférieure à 2 % qui elle est interprétable et a un sens.
Il n'y a donc pas lieu de suspecter une quelconque contamination bactérienne ou une quelconque dégradation de l'échantillon 995474 B à l'examen des résultats qui figurent aux pages 55 et 56 du dossier de contre-analyse.

3/ Incohérence des résultats relatifs à la détermination du T/E et des niveaux de testostérone et d'épitestostérone

Pages 12 à 15 de l'argumentaire de F. Landis, il est fait référence à une première opération de confirmation qui a été jugée non satisfaisante par le laboratoire en raison de la présence d'une inhibition de la dérivation et de son impact sur la valeur du standard interne (Méthyltestostérone). Dans cette expérimentation, la valeur T/E n'est pas altérée puisque sa détermination qui est obtenue par rapport des surfaces des signaux de chacun des composés est indépendante du standard interne. Par contre, les valeurs des concentrations de testostérone et d'épitestostérone sont forcément faussées. Ce résultat d'analyse a été placé en annexe comme le stipule les référentiels mais ne peut en aucun cas être comparé ni à celui de la deuxième opération de confirmation pratiquée sur l'échantillon A ni à celui de l'opération de contre-analyse, du moins en ce qui concerne les niveaux de concentration de testostérone et d'épitestostérone qui sont au demeurant en parfaite cohérence dans les deux dernières opérations pratiquées sur les échantillons A et B.

Pages 16 et 17 de l'argumentaire de F. Landis, il est fait état d'une incohérence des résultats du rapport T/E à partir d'une comparaison des résultats obtenus par deux méthodes différentes sur l'échantillon A (screening et confirmation). La comparaison des résultats obtenus par une méthode sur les échantillons A et B aboutit en fait à des résultats identiques. Une distorsion entre les résultats de screening et de confirmation est classique surtout lorsqu'il s'agit comme dans le cas de l'échantillon 995474 de concentrations d'épitestostérone faibles.

Page 22 de l'argumentaire de F. Landis, il est fait état d'un seuil de 200 ng/mL de testostérone pour déclarer un cas positif. Il s'agit en fait d'un seuil d'alarme supplémentaire au rapport T/E pour déclencher une éventuelle analyse isotopique où un suivi du sportif. Ce seuil ne constitue ni une condition nécessaire ni une condition suffisante pour déclarer un cas positif.

4/ Interprétation des résultats de l'analyse isotopique

Pages 18 à 21 de l'argumentaire de F. Landis, il est fait état que les appauvrissements isotopiques ont été mesurés sur les 4 métabolites et qu'un seul métabolite a été trouvé positif dans l'opération de contre-analyse. Dans les référentiels, un seul métabolite est exigé pour déclarer un cas positif.

www.afld.fr | 143, avenue Roger Salengro 92290 Châtenay-Malabry | Tél : +33 (0)1 46 60 28 60 | Fax : +33 (0)1 46 60 30 17

afld
agence française de lutte contre le dopage
Département des Analyses

5/ Autres arguments

Page 23 de l'argumentaire de F. Landis, Il est fait état de la déclaration des médicaments sur l'exemplaire du formulaire de contrôle antidopage destiné au laboratoire avec remise en cause de l'anonymat.

Page 24 de l'argumentaire de F. Landis, Il fait état d'une erreur dactylographique de l'agence américaine antidopage dans l'un de ses échanges avec le conseil de F. Landis.

Pages 25 et 26 de l'argumentaire de F. Landis, Il s'agit de conclusions percutantes qui n'engagent que leurs auteurs et disculpent F. Landis.

J. de CEAURRIZ
Directeur

www.afld.fr | 143, avenue Roger Salengro 92290 Châtenay-Malabry | Tél : +33 (0)1 46 60 28 60 | Fax : +33 (0)1 46 60 30 17